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ORIGINAL ARTICLE

Nutritional Composition and Antioxidant Activity of Vobla-roach (*Rutilus rutilus caspicus*) Muscle Tissue Exposed to Heavy Metals

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ABSTRACT

The present study was carried out to study the toxic effects of different sublethal concentrations of cadmium chloride and lead salts [$CdCl_2$ and $Pb(NO_3)_2$] on lipid, carbohydrate, protein contents and antioxidant enzymes of vobla-roach (Rutilus rutilus caspicus), a common Caspian fish species, in laboratory bioassays. R. rutilus caspicus was exposed to various sublethal concentrations of $CdCl_2$ and $Pb(NO_3)_2$, i.e. 0.75, 1.5 and 2.25 mg L^{-1} for 24, 48, 72 and 96 hr. Then, the fishes were sacrificed and muscle was collected for the determination of carbohydrates, lipids, proteins, lipid peroxidation (LPO), catalase (CAT) and superoxide dismutase (SOD) activities. The results showed that the maximum decrease in lipid, carbohydrate and protein contents were observed 96 hr after treatment with 2.25 mg L^{-1} of $Pb(NO_3)_2$ (55.76%, 46.25% and 48.81%, respectively). Furthermore, antioxidant enzyme activities increased when fishes were exposed to cadmium and lead. The maximum increase in the lipid peroxidation and superoxide dismutase was recorded at 96 hr after treatment with 2.25 mg L^{-1} of $Pb(NO_3)_2$ (184.47 % and 110.52 %, respectively). According to the results, catalase activity decreased with increasing concentration of heavy metals and exposure duration. The maximum decrease in catalase activity was observed 96 hr after treatment with 2.25 mg L^{-1} of $Pb(NO_3)_2$ (81.21 %). In general, both heavy metals were highly toxic in muscle of R. rutilus caspicus, with lead showing higher toxicity then cadmium in terms of nutritional parameters and antioxidant enzyme activity.

Keywords: Antioxidant enzymes, *Rutilus rutilus caspicus,* nutrients, heavy metals, oxidative stress.

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INTRODUCTION

In living organisms, one of the main mechanisms of toxicity common to many xenobiotics is oxidative stress, i.e. the production and accumulation of molecular oxygen byproducts, the reactive oxygen species (ROS, such as $\cdot O^{2-}$, H_2O_2, $OH^{\cdot}$,$OH\cdot$, $R\cdot$ and $ROO\cdot$), in cells and tissues [1]. ROS damage biomacromolecules and cause lipid peroxidation, protein oxidation, DNA strand breaks, mutations, chromosomal aberrations and carcinogenesis [2, 3]. Anthropogenic contaminants, including heavy metals and organic pollutants, can generate oxidative stress, as well as other xenobiotics such as toxins produced during cyanobacteria massive blooms [4, 5]. Many organisms, including fishes, have developed sophisticated mechanisms to counteract the detrimental effects of ROS, which consist of both non-enzymatic (ascorbic acid, vitamin C; tocopherols, vitamin E; glutathione, GSH; uric acid) and enzymatic (superoxide dismutase, SOD; catalases, CAT, peroxidases) scavengers [6].

Because oxidative stress is mechanistically involved in heavy metal toxicity [7-10], in the last decade, biomarkers of oxidative stress have been developed to investigate the biochemical perturbations in aquatic organisms due to heavy metal exposure [11, 12]. Heavy metals enter the aquatic ecosystem by different routes, including both anthropogenic and natural sources [13-16], representing, currently, a major environmental threat [17]. Fishes are particularly sensitive to the water pollutants [4, 18, 19]. Once absorbed, contaminants may impair biochemical, physiological and reproductive processes, damage

tissues and organs and cause mortality [20]. In addition, fishes can be used for the assessment of the quality of aquatic environments, as bioindicators of environmental pollution [21]. To defend themselves from heavy metal toxicity, fishes possess a series of detoxification enzymes whose activities are modulated by various factors including fish age, nutritional status, water temperature and dissolved oxygen concentration [10, 22-24].

Cadmium (Cd) and lead (Pb) are highly toxic heavy metals, since they cause harmful effects in organisms at low levels of exposure [16, 25]. Therefore, the aim of this study was to investigate the effects of heavy metal contamination on the nutritional composition and antioxidant capacity of the fish vobla-roach (*Rutilus rutilus caspicus).* This cyprinid species, native to Caspian Sea, is relevant for commercial fisheries, though it is considered for listing as a threatened species, due to overfishing and environmental deterioration [26].

MATERIALS AND METHODS

Fish sample preparation

The test animals *Rutilus rutilus caspicus* (vobla-roach) having the length of 20-25 cm and weight of 640-650 g were collected from the Gorgan bay, southeastern Caspian sea, Iran. Fishes were adapted to the laboratory conditions and fed with commercially food. The parameters of the water used were: pH 7.1, temperature 20 °C, DO (dissolved oxygen) 8.9 mg L^{-1}, TDS (total dissolved solids) 0.723 mg L^{-1}, conductivity 1.76 mmho/cm. Water was renewed every 24 hr and, to maintain high oxygen concentration, it was aerated continuously. The 96 hr LC50 values of $Pb(NO_3)_2$ and $CdCl_2$were measured using probit analysis [27] and they were 2.30 mg L^{-1} and 2.35 mg L^{-1} , respectively. Then, sublethal concentrations including 0.75 mg L^{-1}, 1.5 mg L^{-1}, 2.25 mg L^{-1} of $Pb(NO_3)_2$ and $CdCl_2$ were prepared and untreated animals were used as control. Feeding was stopped 24 hr before exposure to heavy metals. Twelve fishes were exposed to each concentration. Three animals were taken out from each container at 24, 48, 72 and 96 hr intervals and their weight and length were measured. Then, fishes were sacrificed and muscles were collected and stored at -20°C until analyses.

Estimation of total carbohydrates

Total carbohydrates were measured by the Carroll et al. method [28]. Muscle tissues (200 mg) were homogenized in distilled water (20 mL) and 5% trichloroacetic acid (TCA) in a homogenizer. Then, the homogenate was centrifuged at 3000 rpm for 15 min and 5 mL of anthrone reagent were added to 0.2 mL of supernatant and boiled for 15 min. The tubes were cooled and absorbance determined at 620 nm in a UV/vis spectrophotometer (model UV - 1650PC, Shimadzu, Japan). Blank consisted in 10% TCA and anthrone (1:1). Results were expressed as mg of glucose g^{-1} fresh weight (FW) and in percentage.

Evaluation of total lipids

Lipids were extracted as described by Folch et al. method [29], and determined by the Barnes and Blackstock method [30]. In brief, 50 mg of muscle tissue were homogenized (5% w/v) in a warming blender in chloroform-methanol mixture (2:1). The homogenates were filtered by Whatman No.1 filter paper, and the residue was again homogenised as before and then filtered. The non-lipid fraction from pooled filtrate was removed through shaking vigorously with 0.88% KCl (added as 1/4 of the volume). One mL of filtrate was evaporated in a test tube under nitrogen and 1 mL of concentrated H_2SO_4 was added and boiled for 10 min. For determination of total lipid, 0.2 mL of solution was added to 5 mL of vanillin reagent. The colour variation was determined in a spectrophotometer at 520 nm against blank.

Assessment of total proteins

Protein was determined following the Lowry et al. method [31]. Briefly, 100 mg of the muscle were homogenized with 5% TCA in a homogenizer. The homogenate was centrifuged at 3000 rpm for 10 min and the residue was dissolved in 0.1N NaOH. An aliquot (0.2 mL) of this solution was diluted to 1 mL using 0.1N NaOH and, then, 3.5 mL of the Folin's reagent were added and mixed. After 30 min, the optical density was determined at 670 nm in a spectrophotometer.

Antioxidant enzyme activity assays

Muscle was excised and homogenized using a chilled pestle and mortar under liquid nitrogen, and, then, it was extracted following the Wang et al. method [32]. The 1.5 mL reaction mixture consisted of 20 mmol L^{-1} sodium phosphate buffer (pH 7.8) and 0.5 mmol L^{-1} ethylene diamine tetraacetic acid (EDTA). Crude extract was obtained after centrifugation at 10000 rpm for 3 min at 4 °C. The supernatant was used for antioxidant enzyme activity assays.

Catalase (CAT) assay - CAT activity was measured by the method described by Montavon et al. [33]. CAT enzyme-activity unit was the amount of extract that caused the decomposition of 1 µmol of H_2O_2 min^{-1} under the experimental conditions. The activity was expressed in U/g FW.

Superoxide dismutase (SOD) assay - SOD activity was determined according to the McCord and Fridovich protocol [34]. The 3 mL reaction mixture consisted of sodium phosphate buffer (50 mmol L^{-1},

pH 7.8), nitroblue tetrazolium (NBT, 75 µmol L^{-1}), methionine (13 mmol L^{-1}), riboflavin (2 µmol L^{-1}) and enzyme extract (50 µL). The reaction mixture was incubated for 15 min in fluorescent light at 25 °C, and absorbance was measured at 560 nm with a spectrophotometer. Non-illuminated mixture without enzyme extract was used as control. The volume of enzyme that corresponded to 50% inhibition of reaction was determined. It was defined as one enzyme-activity unit and the activity was expressed in U/g FW.

***Lipid peroxidation assay* -** This assay was adapted from the thiobarbituric acid reactive substance (TBARS) protocol [35]. Five µL of each sample were added to 45 µL of 50 mM monobasic sodium phosphate buffer. Then, 93.5 µL of TCA (20%, pH 3.5), 12.5 µL of SDS (sodium dodecyl sulfate, 8.1%), 93.5 µL of thiobarbituric acid (1%) and 50.5 µL of Milli-Q grade ultrapure water were added. Mixtures were homogenized in a vortex for 30 s and, then, the tubes' lids were perforated with a needle and the tubes were incubated in boiling water for 10 min. Afterward, tubes were placed in ice for a few minutes to cool and 312.5 µL of *n*-butanol pyridine (15:1, v/v) and 62.5 µL of Milli-Q grade ultrapure water were added. Tubes were placed in a vortex and centrifuged at 7000 rpm for 5 min. Two 150 µL aliquots of the supernatant of each reaction were put into a 96-well microplate and absorbance was determined at 530 nm by a spectrophotometer. To quantify the lipid peroxides, an eight-point calibration curve (0.0 - 0.3 µM TBARS) was calculated using malondialdehyde bis (dimethylacetal) standard (from Merck, Germany).

Statistical Analyses

One-way analysis of variance (ANOVA) was used to analyze the differences intra-groups and inter-groups between. The significance level was $p < 0.05$.

RESULTS AND DISCUSSION

Effect of $Pb(NO_3)_2$ and $CdCl_2$ on carbohydrate content

Prolonged exposure to different sublethal concentrations of heavy metals caused a gradual decrease in the carbohydrate content determined in muscle tissues of *R. rutilus caspicus* (Table 1). The result showed that there was a significant difference, in carbohydrate content, between the exposure duration and different concentrations of Pb and Cd ($p \leq 0.05$) (Table 1). In particular, for $CdCl_2$ and $Pb(NO_3)_2$, the maximum percent decrease was recorded at 2.25 mg L^{-1} after 96 hr of exposure (39.20% and 46.25%, respectively) (Table 1).

Effect of $Pb(NO_3)_2$ and $CdCl_2$ on lipid content

A gradual reduction of the lipid content was observed in muscle of *R. rutilus caspicus* after prolonged exposure to different sublethal concentrations of heavy metals (Table 1). For both Pb and Cd, there was a significant difference between the different concentrations and exposure duration in lipid content ($p \leq 0.05$) (Table 1). The highest decrease for $CdCl_2$ and $Pb(NO_3)_2$ was measured at 2.25 mg L^{-1} after 96 hr of exposure (51.39% and 55.76%, respectively) (Table 1).

Effect of $Pb(NO_3)_2$ and $CdCl_2$ on protein content

The results showed a gradual decrease in protein content of *R. rutilus caspicus* tissues at different sublethal concentrations of heavy metals for various exposure periods (Table 1). According to the results, there was a significant difference between the different concentrations of $CdCl_2$ and $Pb(NO_3)_2$ and exposure duration for protein content ($p \leq 0.05$) (Table 1). For Cd and Pb, the maximum reduction of protein content was recorded at 2.25 mg L^{-1} after 96 hr of exposure (41.20 % and 48.81 % in compare with control (Table 1).

Table 1. Effect of heavy metal [$CdCl_2$ and $Pb(NO_3)_2$] on lipid, carbohydrate and protein content

Exposure (hr)	Concentration (ppm)	Lipid (mg g^{-1})		Carbohydrate (mg g^{-1})		Proteins(mg g^{-1})	
		$CdCl_2$	$Pb(NO_3)_2$	$CdCl_2$	$Pb(NO_3)_2$	$CdCl_2$	$Pb(NO_3)_2$
24	0	57.6 ± 0.11^{s}	57.6 ± 0.11^{s}	23.7 ± 0.11^{k}	23.7 ± 0.11^{k}	38.5 ± 0.28^{q}	38.5 ± 0.28^{q}
	0.75	54.3 ± 0.05^{r} (5.72 %)	53.8 ± 0.46^{qr} (6.59 %)	20.8 ± 0.51^{h} (12.23 %)	19.9 ± 0.51^{h} (16.03 %)	36.4 ± 0.23^{p} (5.45 %)	34.8 ± 0.46^{m} (9.61 %)
	1.5	48.1 ± 0.63^{n} (16.49 %)	47.9 ± 0.51^{n} (16.84 %)	18.2 ± 0.11^{g} (23.20 %)	17.8 ± 0.46^{g} (24.89 %)	34.3 ± 0.17^{lm} (10.90 %)	33.6 ± 0.34^{kl} (12.72 %)
	2.25	41.2 ± 0.05^{k} (28.47 %)	39.7 ± 0.40^{h} (31.07 %)	15.6 ± 0.34^{ef} (34.17 %)	13.9 ± 0.51^{bc} (41.35 %)	31.2 ± 0.11^{g} (18.96 %)	29.7 ± 0.40^{ef} (22.85 %)
48	0	57.5 ± 0.46^{s}	57.5 ± 0.40^{s}	23.6 ± 0.34^{k}	23.6 ± 0.34^{k}	38.2 ± 0.11^{q}	38.2 ± 0.11^{q}
	0.75	53.1 ± 0.05^{pq} (7.65 %)	52.6 ± 0.34^{p} (8.52 %)	20.4 ± 0.23^{h} (13.55 %)	19.8 ± 0.46^{h} (16.10 %)	35.6 ± 0.34^{n} (6.80 %)	34.6 ± 0.34^{m} (9.42 %)
	1.5	46.2 ± 0.46^{m} (19.65 %)	44.8 ± 0.46^{l} (22.08 %)	17.8 ± 0.46^{g} (24.57 %)	15.6 ± 0.34^{ef} (33.89 %)	33.3 ± 0.17^{k} (12.82 %)	32.1 ± 0.05^{h} (15.96 %)
	2.25	38.4 ± 0.23^{g} (33.21 %)	35.2 ± 0.11^{e} (38.78 %)	14.9 ± 0.51^{cde} (36.83 %)	12.3 ± 0.17^{a} (47.88 %)	29.7 ± 0.40^{ef} (22.25 %)	27.4 ± 0.23^{d} (28.27 %)
72	0	57.3 ± 0.17^{s}	57.3 ± 0.17^{s}	22.9 ± 0.51^{k}	22.9 ± 0.51^{k}	38.1 ± 0.05^{q}	38.1 ± 0.05^{q}
	0.75	50.2 ± 0.46^{o}	48.8 ± 0.46^{n}	19.7 ± 0.40^{h}	18.6 ± 0.34^{g}	33.2 ± 0.11^{k}	30.2 ± 0.11^{f}

	1.5	(12.39 %) 41.6±0.23^{k} (27.39 %)	(14.83 %) 39.5±0.28^{h} (31.06 %)	(13.97 %) 16.6±0.34^{f} (27.51 %)	(18.77 %) 14.5±0.28^{cde} (36.68 %)	(12.86 %) 31.1±0.05^{g} (18.37 %)	(20.73 %) 29.3±0.17^{e} (23.09 %)
	2.25	33.7±0.17^{d} (41.18 %)	30.2±0.11^{c} (47.29 %)	14.1±0.05^{bcd} (38.42 %)	13.2±0.11^{ab} (42.35 %)	26.8±0.46^{d} (29.65 %)	24.6±0.34^{c} (35.43 %)
96	0	57.2±0.46^{s}	57.2±0.46^{s}	22.7±0.40^{k}	22.7±0.40^{k}	38.1±0.05^{q}	38.1±0.05^{q}
	0.75	46.1±0.34^{m} (19.40 %)	45.7±0.40^{lm} (20.10 %)	19.8±0.46^{h} (12.77 %)	18.6±0.34^{g} (18.06 %)	31.4±0.23^{gh} (17.58 %)	29.1±0.05^{e} (23.62 %)
	1.5	36.5±0.23^{f} (36.18 %)	34.6±0.34^{de} (39.51 %)	15.2±0.11^{de} (33.03 %)	13.3±0.17^{ab} (41.40 %).	29.2±0.11^{e} (23.35 %)	26.8±0.46^{d} (29.65 %)
	2.25	27.8±0.46^{b} (51.39 %)	25.3±0.17^{a} (55.76 %)	13.8±0.46^{bc} (39.20 %)	12.2±0.11^{a} (46.25 %)	22.4±0.23^{b} (41.20 %)	19.5±0.28^{a} (48.81 %)

*Different letters indicate significant differences ($p < 0.05$) according to Duncan's multiple range test.

Effect of $Pb(NO_3)_2$ and $CdCl_2$ on lipid peroxidation (LPO) activity

LPO activity in muscle tissues of vobla-roach significantly increased with increasing heavy metal concentration and exposure duration ($p \leq 0.05$) (Figure 1). The highest LPO activity was recorded at 2.25 mg L^{-1} of $Pb(NO_3)_2$ after 96 hr of exposure, with 26.94 nM MDA^{-1} mg^{-1} protein (184.47% increase compared to control) (Figure 1).

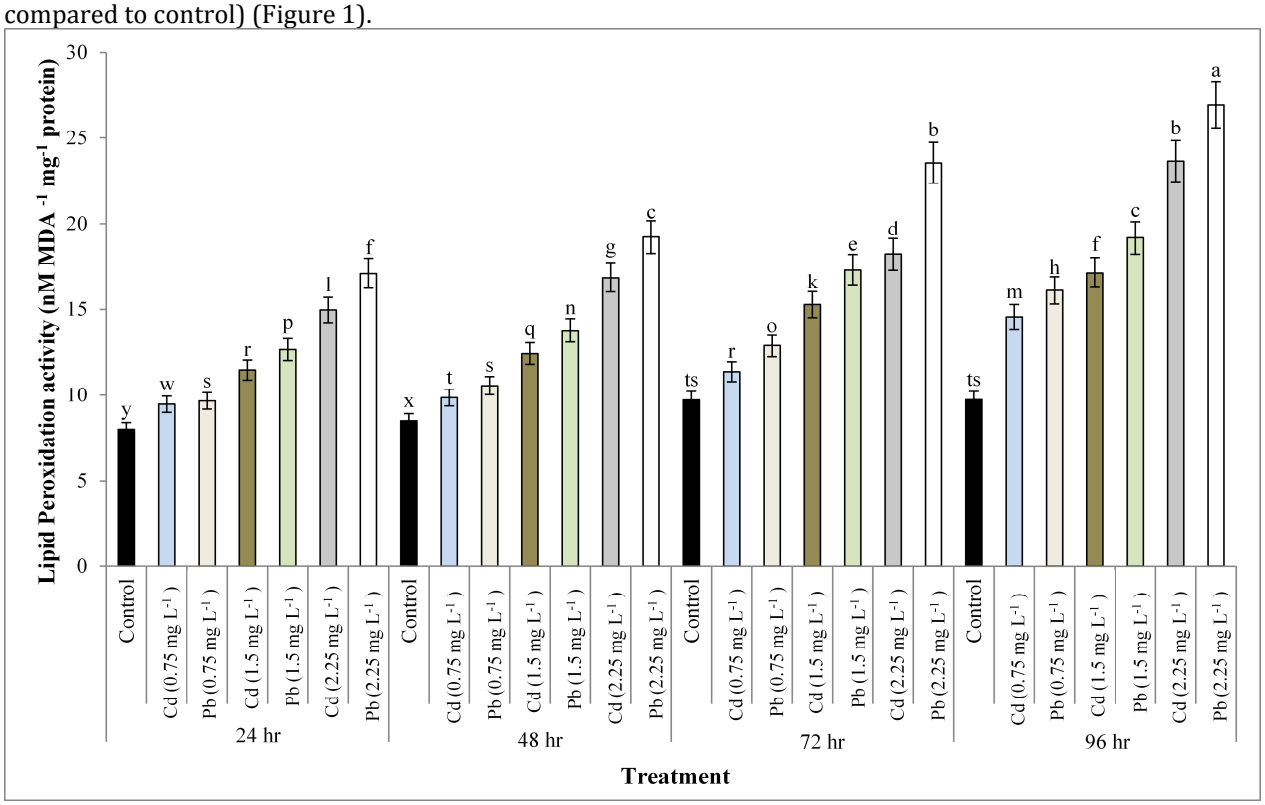

Fig. 1. Lipid peroxidation activity (nM MDA^{-1} mg^{-1} protein) in *Rutilus rutilus caspicus* exposed to different sublethal concentrations of $CdCl_2$ (Cd) and $Pb(NO_3)_2$ (Pb) for 24, 48, 72 and 96 hr. Different letters indicate significant differences ($p < 0.05$) according to Duncan's multiple range test.

Effect of $Pb(NO_3)_2$ and $CdCl_2$ on superoxide dismutase (SOD) activity

When fishes were exposed to different sublethal concentrations of Cd and Pb up to 96 hr, a significant increase in the muscle SOD activity was reported ($p \leq 0.05$) (Figure 2). In control samples, SOD activity levels were 15.8, 16.2, 16.23 and 17.3 U/mg^{-1} protein after 24, 48, 72 and 96 hr of exposure, respectively (Figure 2). The maximum SOD activity was detected for $Pb(NO_3)_2$ at 2.25 mg L^{-1}, after 96 hr, with 36.42 U/mg^{-1} protein (110.52% increase in comparison with control) (Figure 2).

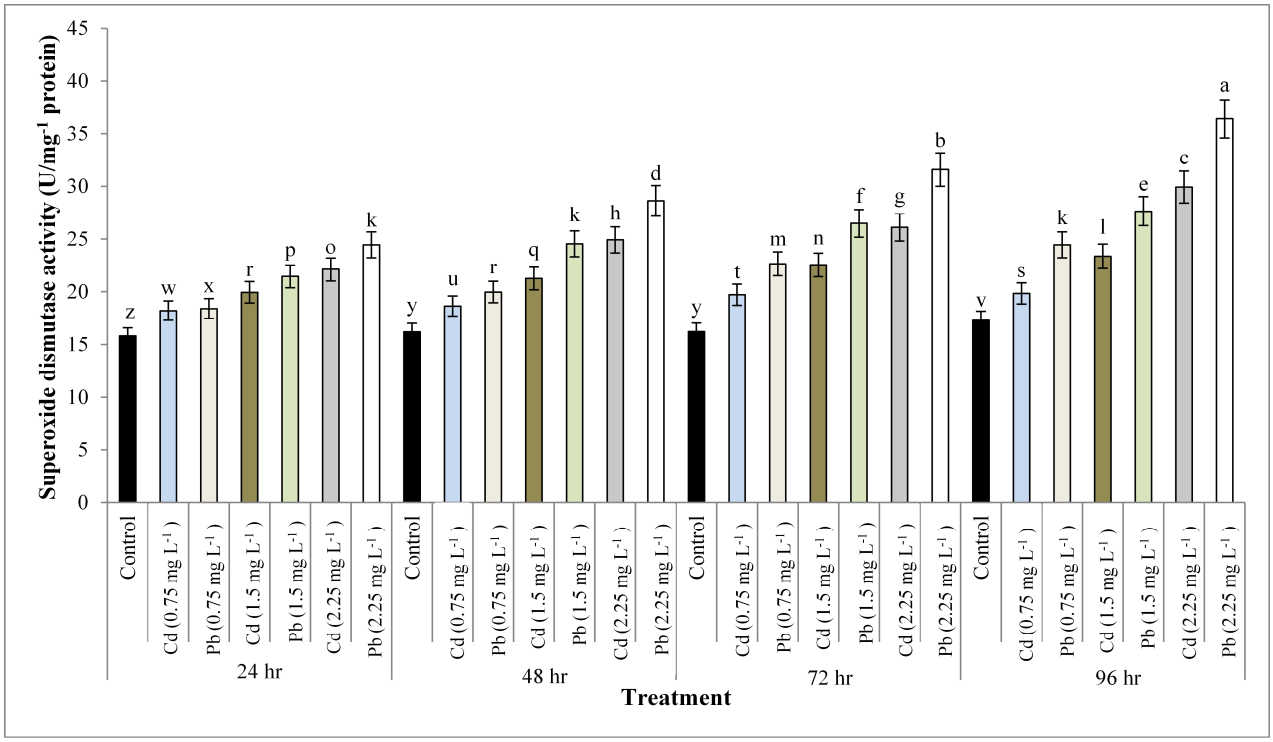

Fig. 2. Superoxide dismutase (SOD) activity (units of SOD mg^{-1} protein) in *Rutilus rutilus caspicus* exposed to different sublethal concentrations of $CdCl_2$ (Cd) and $Pb(NO_3)_2$ (Pb) for 24, 48, 72 and 96 hr. Different letters indicate significant differences ($p < 0.05$) according to Duncan's multiple range test.

Effect of $Pb(NO_3)_2$ and $CdCl_2$ on catalase (CAT) activity

A significant decrease of tissue CAT activity was registered when fishes were exposed to different heavy metal concentrations for different periods ($p \leq 0.05$) (Figure 3). In controls, the CAT activities were 6.4, 6.5, 6.6 and 6.6 nM $mg^{-1}sec^{-1}$ protein 24, 48, 72 and 96 hr after exposure, respectively (Figure 3). The highest decrease in CAT activity was determined for $Pb(NO_3)_2$ at 2.25 mg L^{-1}, after 96 hr, with 1.24 nM $mg^{-1}sec^{-1}$ protein (81.21 % decrease compared with control) (Figure 3).

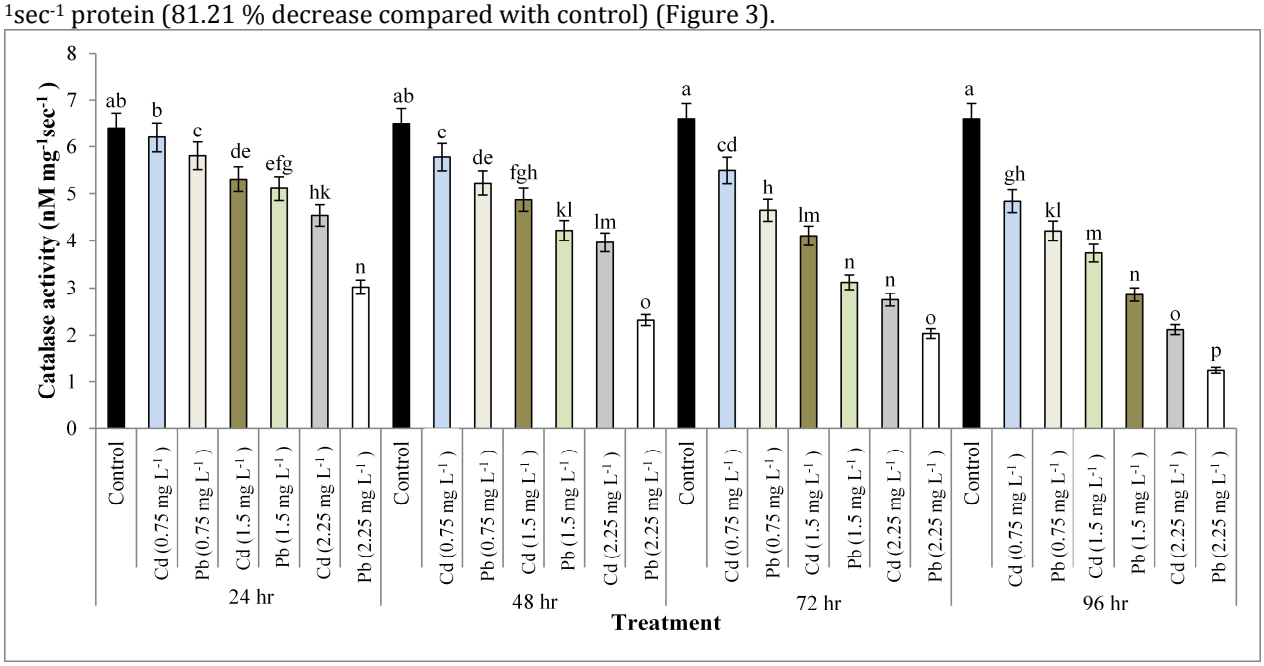

Fig. 3. Catalase activity (nM mg^{-1} sec^{-1} muscle tissue) in *Rutilus rutilus caspicus* exposed to different sublethal concentrations of $CdCl_2$ (Cd) and $Pb(NO_3)_2$ (Pb) for 24, 48, 72 and 96 hr. Different letters indicate significant differences ($p < 0.05$) according to Duncan's multiple range test.

In this study, we showed that exposure of *R. rutilus caspicus* to different sublethal concentrations of Cd and Pb significantly altered the nutritional composition of muscle tissues, by decreasing total carbohydrate, lipid and protein contents. The decrease in protein content may be ascribed to increased

protein turnover rate or to the imbalance of protein biosynthesis caused by toxicants. In addition, cell proteolytic activity was also shown to reduce the protein content [36]. Under stressful conditions, proteins can be consumed as an alternative substrate for generating energy. Therefore, vobla-roach could have used proteins for energy production to mitigate and/or repair the heavy metal damages. These results are in agreement with studies that reported decrease in protein content in various organisms under heavy metal exposure [36-38]. In a hostile environment, fishes mobilize the stored energy to meet the body energy demands through glycogenolysis and gluconeogenesis. Previous findings suggested that when animals are subjected to toxic pollutants, metabolism of carbohydrate is disturbed [39, 40]. Consequently, rapid glycogen transferase activation and glycogenolysis, as well as reduced intestinal absorption of nutrients, could have caused the decrease of carbohydrates [41]. Lipids are one of the most important energy sources and structural components in animals. In particular, in aquatic organisms, lipids represent an important energetic reserve too, rapidly metabolized to meet the body energy needs. Reduction in lipid content might result from lipolysis or mitochondrial injury which impairs tricarbossilic (Krebs) acid cycle and fatty acid oxidation [41].

Oxidative stress occurs when the critical balance between oxidants and antioxidants is disturbed as a result of antioxidant depletion or ROS accumulation or both, leading to cellular damage [42, 43]. As previously introduced, oxidative stress in mechanistically involved in toxicity of heavy metals, which, in turn, stimulate the cell antioxidant defence mechanisms [44]. In particular, heavy metals damage mitochondria and deplete the cell pool of GSH, a powerful antioxidant, thus generating ROS accumulation and oxidative stress [45, 46]. These highly reactive O_2 by-products may damage proteins, lipids, carbohydrates and nucleic acids and may induce physiological, biochemical and metabolic alterations in fish tissues [47, 48].

Our results showed that exposure to Cd and Pb increased LPO and SOD activities in muscle tissues of vobla-roach, possibly due to ROS generation in muscle tissues. In aerobic organisms, the cell SOD-CAT system provides the first defense to counteract ROS toxicity. SOD converts superoxide anion ($\cdot O_2^-$) into hydrogen peroxide (H_2O_2) and represents one of the most responsive indicator of exposure to contaminants eliciting oxidative stress [11].

CAT detoxifies H_2O_2 in peroxisomes. In our experimental conditions, CAT activity decreased in pollutant exposed fishes, even if increased SOD activity, presumably, produced high levels of this ROS. Therefore, it is possible that H_2O_2, the most diffusible ROS, accumulated in cytoplasm, where it was scavenged by different enzymes, such as GSH-peroxidases [6, 49].

In agreement with previous studies, our results showed that Cd ad Pb exposure caused high levels of lipid peroxides in fish tissues. Heavy metal-induced lipid peroxidation was documented in many studies [10, 50, 51]. When the cell scavengers are inadequate to detoxify ROS, injury to cell structures may occur, and one of the most serious damages is lipid peroxidation of membranes [52].

CONCLUSIONS

Heavy metal pollution of fresh waters is known to disturb the sensitive equilibrium of the aquatic ecosystem. Among the different toxic pollutants, heavy metals are particularly harmful because of their biomagnification in the food chain. They rapidly concentrate in various fish organs, particularly in lipid tissues, resulting in biomagnification to toxic levels even when the exposure is low. The present study indicated that sublethal concentrations of Cd and Pb altered the normal metabolism of the freshwater fish *Rutilus rutilus caspicus.* In addition, metal exposure was associated to exacerbated oxidative stress conditions and impaired antioxidant defense system of this Caspian fish species. The results of this study suggest that enzymatic biomarkers of oxidative stress may be sensitive indicators of aquatic pollution, suggesting that antioxidant enzymes may be used as Cd and Pb toxicity biomarkers and fish species may serve as bioindicators of heavy metal pollution.

Conflict of interest

Authors declare no conflict of interest

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