Taylor & Francis
Taylor & Francis Group

Human and Ecological Risk Assessment: An International Journal

ISSN: 1080-7039 (Print) 1549-7860 (Online) Journal homepage: http://www.tandfonline.com/loi/bher20

Investigation of Cadmium-Induced Genotoxicity and Oxidative Stress Response in Indian Major Carp, Labeo rohita

Anurag Dabas , N. S. Nagpure , R. M. Mishra , Basdeo Kushwaha , Ravindra Kumar & Pavan Kumar

To cite this article: Anurag Dabas , N. S. Nagpure , R. M. Mishra , Basdeo Kushwaha , Ravindra Kumar & Pavan Kumar (2014) Investigation of Cadmium-Induced Genotoxicity and Oxidative Stress Response in Indian Major Carp, Labeo rohita , Human and Ecological Risk Assessment: An International Journal, 20:2, 510-526, DOI: 10.1080/10807039.2012.702591

To link to this article: http://dx.doi.org/10.1080/10807039.2012.702591

Accepted author version posted online: 09 Jul 2012.
Published online: 09 Jul 2012.

Human and Ecological Risk Assessment, 20: 510–526, 2014

ISSN: 1080-7039 print / 1549-7860 online
DOI: 10.1080/10807039.2012.702591

Investigation of Cadmium-Induced Genotoxicity and Oxidative Stress Response in Indian Major Carp, *Labeo rohita*

Anurag Dabas,[1] N. S. Nagpure,[1] R. M. Mishra,[2] Basdeo Kushwaha,[1] Ravindra Kumar,[1] and Pavan Kumar[1]
[1]Molecular Biology and Biotechnology Division, National Bureau of Fish Genetic Resources, Lucknow, India; [2]Department of Environmental Biology, Awadhesh Pratap Singh University, Rewa, Madhya Pradesh, India

ABSTRACT

We investigated genotoxicity and oxidative stress in the gills of *Labeo rohita* exposed to 33.6, 67.1, and 100.6 mg L^{-1} of cadmium chloride at 96 h. Genotoxicity was assessed using single cell gel electrophoresis whereas oxidative stress was monitored through lipid peroxidation induction and antioxidant response parameters, namely reduced glutathione (GSH), glutathione peroxidase, glutathione-S-transferase, superoxide dismutase, and catalase (CAT) activities. Significant ($p < .05$) effect of both concentration and time of exposure was observed on the extent of DNA damage in treated fish. Similarly, malondialdehyde content, level of GSH, and activities of antioxidant enzymes were significantly elevated in treated groups, except CAT. The increased DNA damage and lipid peroxidation (LPO) content along with fluctuation in antioxidant defense system in fish indicated the interaction of cadmium (Cd) with DNA repair processes and production of reactive oxygen species. Thus, Cd is liable for induction of LPO, alteration of antioxidant defenses, and DNA damage in gills of *L. rohita*.

Key Words: cadmium, genotoxicity, *Labeo rohita*, oxidative stress, antioxidant enzymes.

INTRODUCTION

A variety of contaminants, including toxic heavy metals, are reported to be ubiquitously present in rivers and reservoirs and produce adverse effects on the ecological balance of the recipient environment and harm to a diversity of aquatic organisms (Farombi *et al*. 2007). Cadmium (Cd) is considered as one of the most toxic

Received 14 February 2012; revised manuscript accepted 17 April 2012.
Address correspondence to Basdeo Kushwaha, Molecular Biology and Biotechnology Division, National Bureau of Fish Genetic Resources, Lucknow 226002 (U.P.), India. E-mail: basdeo.scientist@gmail.com

transition elements and a potent industrial hazard, arising primarily from battery, electroplating, pigment, plastics, and fertilizer industries. The dispersion of Cd in the environment has increased in past decades due to its widespread industrial use. Numerous field studies have shown varying degrees of contamination of aquatic systems, via direct or indirect Cd inputs (Hutton 1983; Guinee *et al*. 1999). Lungs, gills, liver, and kidneys are the principal targets of Cd exposure. Moreover, Cd is well known for other various adverse effects (*e.g.*, disturbances of enzyme functions, enhancement of lipid peroxidation influence on mitochondrial functions, and DNA strand break) (Tsuzuki *et al*. 1994; Almeida *et al*. 2002).

Cadmium has many cellular impacts. It triggers induction of some proto-oncogenes, inhibits the DNA repair system, and alters the activity of several enzymes involved in oxidative stress responses (Waisberg *et al*. 2003; Giaginis *et al*. 2006). The mechanism responsible for the genotoxicity of Cd may involve the direct interaction of Cd^{2+} with DNA through the binding of Cd^{2+} at guanine, adenine, and thymine bases (Hossain and Huq 2002). Moreover, Cd acts as a mutagen primarily by direct inhibition of an essential DNA mismatch repair, resulting in a high level of genetic instability (Jin *et al*. 2003). Many studies have highlighted the potential role of active oxygen species and free radicals as mediators in the induction of genotoxicity by Cd (Hartwig 1994; Beyersmann and Hechtenberg 1997). Cadmium also has cytotoxic, carcinogenic, and mutagenic effects in test organisms (Monteiro *et al*. 2007; Cambier *et al*. 2010). Cadmium's cellular toxicity and genotoxicity may also be mediated indirectly; cells under oxidative stress display various dysfunctions due to lesions caused by reactive oxygen species to lipids, proteins, and DNA (Risso-de Faverney *et al*. 2001).

The genotoxic effects of environmental pollutants can be monitored using a broad range of both *in vitro* and *in vivo* biomarker assays. However, the comet assay (or single cell gel electrophoresis assay) is gaining popularity over others because it can be performed quickly and provides increased sensitivity for detecting low levels of DNA damage (Nagpure *et al*. 2007; Nwani *et al*. 2010). As a result, the comet assay is being employed to assess the genotoxicity of various chemical compounds in several test organisms (Talapatra *et al*. 2006; Cavas and Konen 2007; Ali *et al*. 2008; Kumar *et al*. 2010).

Reactive oxygen species (ROS) like superoxide radicals, hydrogen peroxide, and hydroxyl radicals are continuously being formed during normal aerobic metabolism. These react with cellular components resulting in protein oxidation, oxidative DNA damage, and lipid peroxidation (Vutukuru *et al*. 2006; Li *et al*. 2011). Lipid peroxidation (LPO) as induced by heavy metal pollution in aquatic organisms is expressed by malondialdehyde (MDA) formation, which represents the secondary LPO product in the thiobarbituric acid reactive substances (TBARS) test (Draper *et al*. 1993). ROS are detoxified as antioxidant defense systems including antioxidants like reduced glutathione and a set of antioxidant enzymes such as glutathione peroxidase, glutathione-S-transferase, superoxide dismutase, and catalase that protect macromolecules against oxidative damage (Ozmen *et al*. 2004). Exposure to contaminants, including Cd, in aquatic ecosystems can enhance intracellular formation of ROS, which could cause oxidative damage to biological systems (Ferreira *et al*. 2005). Changes in the level of antioxidant enzyme activities can be used as

biomarkers of possible oxidative damage in different aquatic organisms (Livingstone 2003; Bechard *et al*. 2008).

Labeo rohita is a fish of the carp family SouthAsia and SoutheastAsia. It is the most important among the three Indian major carp species used in carp polyculture systems and it has high consumer preference in India. *L. rohita* can serve as an excellent indicator of water quality and environmental pollution in aquatic systems (Vutukuru *et al*. 2007). The gills are the main target of direct contact with contaminants and play a role in metals' uptake, storage, and transfer to the internal compartments via blood transport. Only limited information is available on Cd-induced DNA damage, LPO induction, and alteration in enzymatic as well as non-enzymatic antioxidant activities in gill cells of *L. rohita*. Thus, the present study investigated oxidative stress and genotoxic effects of Cd in gills of the Indian major carp *L. rohita*.

METHODS AND MATERIALS

Experimental Animal and Chemical

Freshwater fish *L. rohita* with similar age and relatedness were procured from a local outlet. After transportation to our laboratory, the specimens were given prophylactic treatment by bathing them twice in 0.05% $KMnO_4$ solution for 2 min to avoid any dermal infections. They had an average (±SD) wet weight and length of 21.0 ± 2.5 g and 13.0 ± 1.2 cm, respectively. They were then acclimatized for 1 month under laboratory conditions before the start of the experiment. Every effort, as suggested by Bennett and Dooley (1982), was made to maintain optimal conditions during acclimatization.

For the present study, technical-grade admium chloride ($CdCl_2$) was procured and used for the study. The chemical was in a monohydrate form, A. R. ($CdCl_2$; 98.0% EC, Maximum limits of impurities, Iron 0.0005% and Sulphate 0.005%) and was manufactured by HiMedia Laboratories Pvt. Ltd., Mumbai, India.

Determination of Exposure Concentrations for Assessing Genotoxicity and Biomarker Response

Exposure concentrations of $CdCl_2$ for use in the assessment of genotoxicity and biomarker response were determined by first conducting acute toxicity bioassays for the test chemical. These were conducted in static systems to determine the 96 h LC_{50} in the test species *L. rohita*. For these tests, 10 acclimatized fish specimens per $CdCl_2$ concentration were randomly exposed for 96 h to test concentrations of 100, 110, 120, 130, 140, 150, 160, and 170 mg L^{-1} along with a control set of fish that was exposed to tap water. The experiment was repeated twice to obtain the 96 h LC_{50} value of the test chemical for the species.

The 96 h LC_{50} value of $CdCl_2$ for freshwater carp *L. rohita* was estimated as 134.18 mg L^{-1}. Three exposure test concentrations for assessing biomarker response were selected as fractions of the 96 h LC_{50} value: low (SL-I; 1/4th of LC_{50} = 33.6 mg L^{-1}), medium (SL-II; 1/2th of LC_{50} = 67.1 mg L^{-1}), and high (SL-III; 3/4th of LC_{50} = 100.6 mg L^{-1}).

In Vivo Exposure Experiment for Assessment of Genotoxicity and Biomarker Response

Healthy fish with average (±SD) wet weight (21.0 ± 2.5 g) and length (13.0 ± 1.2 cm) was randomly selected and 20 fish transferred to each aquarium in 50 L of water. The exposures were continued up to 96 h and tissues were sampled at 24, 48, 72, and 96 h at the rate of five specimens per sampling duration. Fish maintained in water without the test chemical were used as the control. This study was conducted following the OECD guideline No 203 in the static test conditions (OECD 1992).

On each sampling day, gill tissues were collected from fish for single cell gel electrophoresis assay and biochemical investigations. Temperature, pH, conductivity, dissolved oxygen, chloride, total hardness, and total alkalinity were determined for water on each sampling day by standard methods (APHA 2005).

Genotoxicity Assay: Single Cell Gel Electrophoresis (SCGE)

The alkaline single cell gel electrophoresis or comet assay (CA) was performed as a three-layer procedure (Singh *et al*. 1988) with slight modifications (Klaude *et al*. 1996). Gill tissue was removed with the help of scissors and 100 mg tissue was homogenized in an ice-cold homogenization buffer (1× Hanks' balanced salt solution [HBSS], 20 mM EDTA, 10% dimethyl sulfoxide [DMSO], pH 7.0–7.5) followed by centrifugation at 3000 rpm at 4°C for 5 min. The cell pellet was then suspended in chilled phosphate buffered saline (PBS). Viability of gill cells was evaluated by the trypan blue exclusion test method (Anderson *et al*. 1994) and the cell suspensions showing viability >84% were further processed for SCGE according to the method described by Ali *et al*. (2008).

Two slides per specimen were prepared and 20 cells per slide (200 cells per concentration) were scored randomly and analyzed using an image analysis system (Komet-5.5 Kinetic Imaging, UK) attached to a fluorescent microscope (Leica) equipped with appropriate filters. The parameter selected for quantification of DNA damage was percent tail DNA (% tail DNA = 100–% Head DNA), as determined by the imaging software (Komet-5.5 Kinetic Imaging, UK).

Biochemical Assays

Preparation of post-mitochondrial supernatant (PMS)

Gills tissue of 200 mg weight was homogenized in chilled sodium phosphate buffer (0.1 M, pH 7.4) using a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 10,000 g for 10 min at 4°C using a Sigma refrigerated centrifuge (model 3K30) and the resulting supernatant was centrifuged again at 12,500 g for 10 min at 4°C to prepare PMS, which was further used for various biochemical analyses.

Lipid peroxidation (LPO)

Tissue LPO was measured using the method of Ohkawa *et al*. (1979). Tissue homogenate was incubated with 8.1% SDS (w/v) for 10 min followed by addition of 20% acetic acid (*p*H 3.5). The reaction mixture was incubated with 1.2% thiobarbituric acid (w/v) for 1 h in boiling water bath. The reaction product was extracted in

butanol-pyridine solution and read at 532 nm. The amount of TBARS was calculated using a molar extinction coefficient of 1.56×10^5/M per cm. The extent of LPO was expressed as nM TBARS formed/h/mg protein.

Reduced glutathione (GSH)

GSH concentration was measured in gill tissue using the method of Ellman (1959). Samples were treated with trichloroacetic acid (TCA, 10% w/v) and centrifuged at 8900 rpm for 10 min. The supernatant (50 μL) was mixed with Tris-HCl buffer (230 μL, 0.8 M Tris/HCl, 0.02 M EDTA, pH 8.9) and 20 μL of 0.01 M DTNB (2,2′-dinitro-5,5′-dithiobenzoic acid, Ellman′s reagent). The reaction mixture was incubated for 5 min at room temperature. The absorbance of GSH-DNTB conjugate was determined at 412 nm and the concentration (nM GSH/mg protein) was calculated using standard calibration.

Glutathione peroxidase (GPx)

The GPx activity was measured using the procedure of Flohe and Gunzler (1984). One mL of reaction mixture was prepared that contained 0.3 mL of phosphate buffer (0.1 M, pH 7.4), 0.2 mL of GSH (2 mM), 0.1 mL of azide (10 mM), 0.1 mL of H_2O_2 (1 mM), and 0.3 mL of tissue supernatant. After incubation at 37°C for 15 min, reaction was terminated by addition of 0.5 mL of 5% TCA. Tubes were centrifuged at 3000 × g for 5 min and supernatant was collected. 0.2 mL phosphate buffer (0.1 M, pH 7.4) and 0.7 mL of DTNB (0.4 mg/mL) was added to 0.5 mL of reaction supernatant. After mixing, absorbance was recorded at 420 nm. The GPx activity was expressed as nM GSH consumed/min/mg protein.

Glutathione S-transferase (GST)

The activity of GST was determined spectrophotometrically at 25°C by following the formation of GSH conjugate with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm using extinction coefficient of 9.6 mM^{-1} cm^{-1} (Habig *et al*. 1974). The reaction mixture contained 3 mL volume of: 0.1 M sodium phosphate buffer, pH 6.5; 30 mM GSH; 30 mM CDNB in ethanol and the enzyme solution. GST activity was expressed as nM CDNB conjugates/min/mg of protein.

Superoxide dismutase (SOD)

Tissue SOD activity was analyzed using the method of Kakkar *et al*. (1984). The reaction mixture contained 1.2 mL (0.052 mM) sodium pyrophosphate buffer, 0.1 mL (186 mM) phenazine methosulphate, and 0.3 mL (300 mM) nitro blue tetrazolium. Reaction was initiated by adding 0.2 mL NADH (780 mM) and stopped by adding 1 mL glacial acetic acid. Color intensity of the chromogen was measured at 560 nm. The SOD activity was expressed as units/min/mg of protein.

Catalase (CAT)

The activity of CAT was measured according to the method of Aebi (1984). The reaction mixture (3 mL volume) contained: 1.98 mL of 50 mM sodium phosphate buffer (pH 7.0), and 1 mL of 30 mM H_2O_2. The reaction was initiated by adding

20 μL of enzyme extract. The CAT activity was determined by measuring the rate of disappearance of H_2O_2 at 240 nm for 1 min. Enzyme activity was expressed as μM H_2O_2 decomposed/min/mg protein.

Protein estimation

Protein content in tissue samples was determined using a colorimetric method and BSA as standard (Lowry *et al*. 1951).

Statistical Analyses

The 96-h LC_{50} for $CdCl_2$ was calculated using IBM SPSS Statistics (version 19.0). One-way analysis of variance (ANOVA) was used to compare the means obtained for various parameters among different concentrations and durations. A *p*-value less than .05 was considered statistically significant. The values for all the biomarkers are expressed as mean $\pm$ SE (n = 5). Regression analysis was performed to analyze relationships between genotoxicity and various oxidative stress response parameters by using MS Excel.

RESULTS

Physicochemical Properties of the Test Solution

The physicochemical characteristics of the test water measured during experimentation were: temperature 28.6–29.4°C, dissolved oxygen 6.6–7.2 mg L^{-1}, and pH 7.4–7.6. The conductivity of the water ranged from 252–284 μM cm^{-1} while the chloride, total hardness, and total alkalinity varied from 42–48, 160–170, and 220–240 mg L^{-1} as $CaCO_3$, respectively.

DNA Damage

DNA damage was measured as % tail DNA in gill cells (Figure 1A–C) and indicated that fish exposed to different concentrations of $CdCl_2$ exhibited significantly higher DNA damage than control specimens. DNA damage in gill tissue was dose and time dependent. The highest level of DNA damage was observed at SL-III concentration (20.68 $\pm$ 0.59% tail DNA), followed by SL-II (17.24 $\pm$ 0.51% tail DNA), and SL-I concentrations (14.51 $\pm$ 0.45% tail DNA) at 96 h post exposure. Lower exposure concentrations or durations resulted in lower amounts of DNA damage (statistically significant at $p < .05$). The lowest level of DNA damage in gills was observed at 24 h. There was a gradual non-linear increase in the level of DNA damage with progression of the experiment.

Lipid Peroxidation in Gills

LPO in gill cells increased significantly from 2.57 to 3.78 nM TBARS formed/h/mg protein ($p < .05$) at different treatment concentrations of $CdCl_2$ when compared with the control group (Figure 2A). The maximum increase of LPO was observed at SL-III concentration of $CdCl_2$ after 96 h post exposure. The amount of

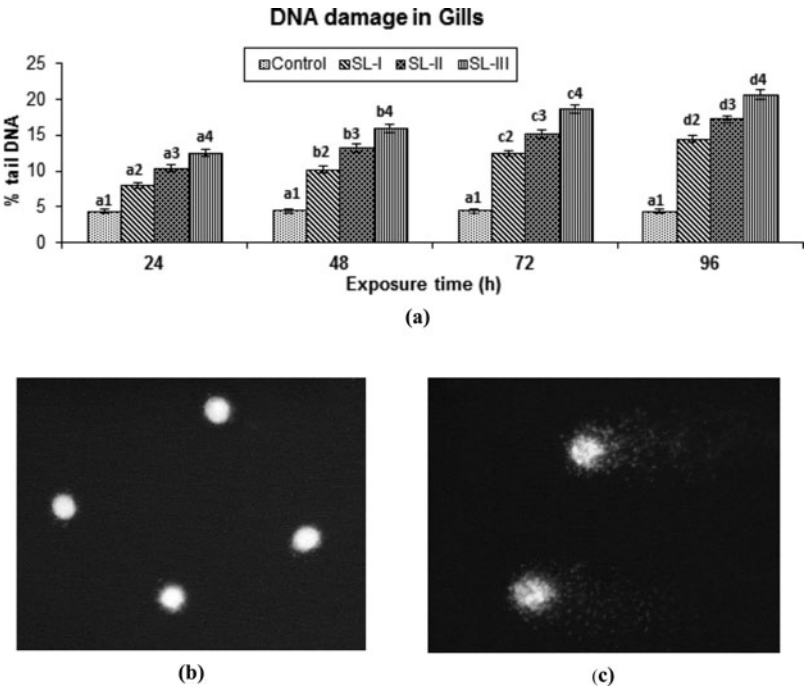

Figure 1. (A) DNA damage in gills of *L. rohita* due to $CdCl_2$ exposure. Values with different superscript letters (lowercase) differ significantly between exposure durations within concentration. Values with different numeric superscripts differ significantly between concentrations within exposure duration. (B) Control gill cells. (C) Cadmium-exposed gill cells at highest concentration and 96 h post exposure.

LPO in gills of treated groups increased significantly with exposure concentration and time.

Induction of Reduced Glutathione

Exposure of *L. rohita* to $CdCl_2$ for 48, 72, and 96 h caused significant ($p < .05$) increase in GSH level in the gills at all concentrations as compared to controls (Figure 3). GSH level in gill tissue was dose-dependent with the highest GSH level observed at SL-III concentration (5.11 ± 0.29 nM GSH/mg protein) at 96 h post exposure. No significant effects among durations of exposure were observed in fish specimens exposed except at the SL-III test concentration and 96 h post exposure.

Antioxidant Enzymes Activities

Significant ($p < .05$) effect of GPx activities was observed in gills of *L. rohita* at all three test concentrations of $CdCl_2$ as compared to control (Table 1). The highest activities of GPx in gill tissue (44.41 ± 2.05 nM GSH consumed/min/mg protein) were observed at the SL-III concentration and 96 h post exposure. Significantly less GPx activity was observed at the SL-II (medium) concentration and 24 h post

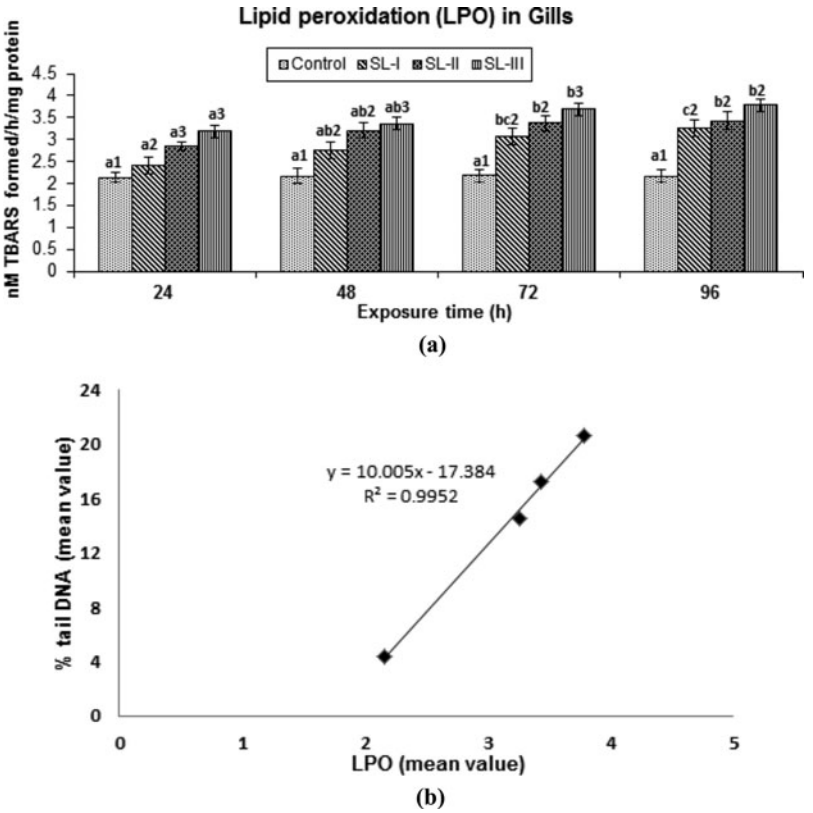

Figure 2. (A) Lipid peroxidation induced by $CdCl_2$ in gills of *L. rohita*. The values are expressed in nanomoles of TBARS formed/h/mg of protein. Values with different superscript letters (lowercase) differ significantly between exposure durations within concentration. Values with different numeric superscripts differ significantly between concentrations within exposure duration. (B) Regression analysis of mean data of lipid peroxidation (LPO) and % tail DNA in fish exposed to $CdCl_2$. Values comprise control and exposed groups at 96 h post-exposure.

exposure. GPx activity in gills of treated groups increased in a dose-dependent manner.

Cadmium chloride induced a significant ($p < .05$) increase in GST activity at most test concentrations and exposure durations; significant increase did not occur for the SL-I concentration and 24 h post exposure (Table 1). The highest increased GST activity was observed in gills (234.04 ± 8.58 nM CDNB conjugates/min/mg of protein) at the SL-III concentration of $CdCl_2$ and 96 h post exposure. No significant effect among durations of exposure was observed in GST activity except at 96 h post exposure and the SL-III test concentration.

A significant ($p < .05$) elevation in SOD activities was observed at all three test concentrations of $CdCl_2$ as compared to control group except at the SL-I concentration

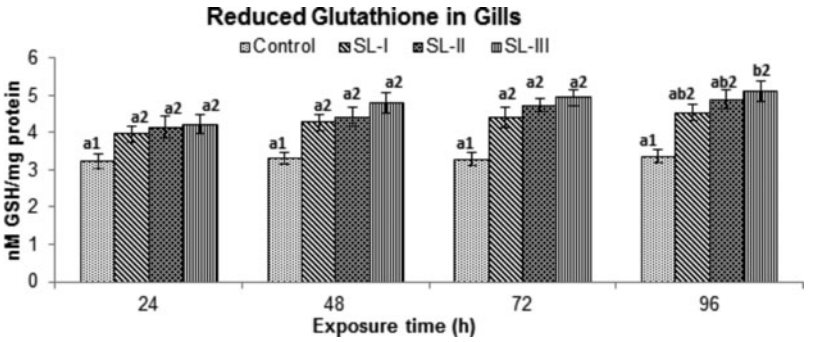

Figure 3. Reduced glutathione level in gill tissues of *L. rohita* as influenced by $CdCl_2$ exposure. The values are expressed in nanomoles of GSH/mg of protein. Values with different superscript letters (lowercase) differ significantly between exposure durations within concentration. Values with different numeric superscripts differ significantly between concentrations within exposure duration.

and 24 h post exposure (Table 2). SOD activity in gills of *L. rohita* increased significantly from 4.02 to 5.32 units/min/mg of protein with highest elevation at SL-III and 96 h post exposure. SOD activity increased with dose and exposure duration.

CAT activity in gills decreased by 171.69 to 141.29 μM H_2O_2 decomposed/min/mg protein at different treatment concentrations of $CdCl_2$ when compared with control group. The smallest significant ($p < .05$) decrease in CAT activity was observed at the SL-III concentration and 24 h post exposure (Table 2). The CAT activity in gills depleted in a dose-dependent manner and highest depletion observed

Table 1. Effect of $CdCl_2$ on glutathione peroxidase (nM GSH consumed/min/mg protein) and glutathione-S-transferase (nM CDNB conjugates/min/mg of protein) enzyme activities in gills of *L. rohita*.

Antioxidant enzymes	Conc.	Exposure time (h)			
		24	48	72	96
GPx	Control	27.16 ± 1.71^{a1}	28.35 ± 1.41^{a1}	29.73 ± 1.58^{a1}	28.84 ± 1.36^{a1}
	SL-I	30.84 ± 1.18^{a1}	34.39 ± 1.65^{ab2}	36.57 ± 1.87^{ab2}	39.04 ± 2.16^{b2}
	SL-II	34.31 ± 1.46^{a2}	36.75 ± 1.95^{ab23}	39.28 ± 1.74^{ab2}	42.73 ± 1.87^{b2}
	SL-III	37.86 ± 1.10^{a2}	40.28 ± 1.32^{ab3}	41.62 ± 2.15^{ab2}	44.41 ± 2.05^{b2}
GST	Control	167.79 ± 4.59^{a1}	168.41 ± 6.56^{a1}	166.92 ± 5.87^{a1}	165.62 ± 6.09^{a1}
	SL-I	187.09 ± 8.31^{a12}	195.38 ± 5.35^{a2}	205.46 ± 8.01^{a2}	211.66 ± 7.44^{a2}
	SL-II	196.75 ± 9.45^{a2}	204.76 ± 9.86^{a2}	211.74 ± 7.39^{a2}	218.14 ± 5.95^{a2}
	SL-III	207.36 ± 7.60^{a2}	215.19 ± 7.78^{ab2}	226.44 ± 8.10^{ab2}	234.04 ± 8.58^{b2}

Values with different superscript letters (lowercase) differ significantly between exposure durations within concentration. Values with different numeric superscripts differ significantly between concentrations within exposure duration.

Table 2. Effect of $CdCl_2$ on superoxide dismutase (units/min/mg of protein) and catalase (μM H_2O_2 decomposed/min/mg protein) enzyme activities in gills of *L. rohita*.

Antioxidant enzymes	Conc.	Exposure time (h)			
		24	48	72	96
SOD	Control	3.18 ± 0.14^{a1}	3.26 ± 0.16^{a1}	3.32 ± 0.20^{a1}	3.29 ± 0.21^{a1}
	SL-I	3.69 ± 0.20^{a12}	3.91 ± 0.21^{ab2}	4.19 ± 0.25^{ab2}	4.43 ± 0.25^{b2}
	SL-II	4.02 ± 0.24^{a2}	4.26 ± 0.25^{ab2}	4.61 ± 0.22^{ab2}	4.95 ± 0.27^{b2}
	SL-III	4.25 ± 0.23^{a2}	4.62 ± 0.22^{ab2}	4.89 ± 0.28^{ab2}	5.32 ± 0.30^{b2}
CAT	Control	184.38 ± 6.16^{a1}	182.13 ± 4.84^{a1}	180.92 ± 3.91^{a1}	183.45 ± 4.35^{a1}
	SL-I	171.69 ± 4.69^{a12}	164.85 ± 6.01^{ab12}	157.42 ± 5.14^{ab2}	151.96 ± 3.15^{b2}
	SL-II	165.82 ± 6.32^{a12}	157.74 ± 4.15^{ab2}	152.45 ± 4.32^{ab2}	147.94 ± 4.08^{b2}
	SL-III	161.69 ± 5.66^{a2}	153.51 ± 3.22^{a2}	148.59 ± 3.51^{ab2}	141.29 ± 3.67^{b2}

Values with different superscript letters (lowercase) differ significantly between exposure durations within concentration. Values with different numeric superscripts differ significantly between concentrations within exposure duration.

at the SL-III test concentration and 96 h post exposure. A significant depletion among exposure durations was observed at 96 h post exposure.

Regression Analysis

Regression analysis (Figure 2B) showed a significant positive correlation ($r^2 = 0.995$) between mean values of% tail DNA and LPO data at 96 h post exposure. This shows that there exists a relationship between genotoxic and oxidative stress-inducing effects of Cd and oxidative stress might be contributing to gentoxicity of Cd in *L. rohita*.

Significant positive correlations were estimated between GSH and both glutathione dependent antioxidant enzyme GPx ($r^2 = 0.999$) and GST ($r^2 = 0.987$) at 96 h post exposure (Figures 4A and 4B). Regression analysis between reduced glutathione and both enzymes (GPx and GST) indicates that GSH has a role in detoxification mechanism along with GPx and GST. A negative correlation ($r^2 = 0.9595$) between SOD and CAT shows that Cd might be directly responsible for suppressing CAT activity and production of free radicals (Figure 4C).

DISCUSSION

The present study investigated genotoxicity and oxidative stress responses in the gill of *L. rohita* exposed to Cd. In aquatic organisms, the gill is the osmoregulatory surface tissue and primary site of uptake of waterborne pollutants and it is the major target organ for acute copper, zinc, cadmium, and silver toxicity to freshwater fish (Karlsson-Norrgren *et al*. 1985; Olsson *et al*. 1998). Failure of the gill to function during acute exposure to Cd can lead to the death of fish. Previous studies show that Cd possibly moves across the epithelial layer of fish gills via calcium channels (Farag *et al*. 1994). Calcium exerts considerable control over permeability of the gills and displacement of calcium could stimulate ion loss and water uptake (Verbost *et al*.

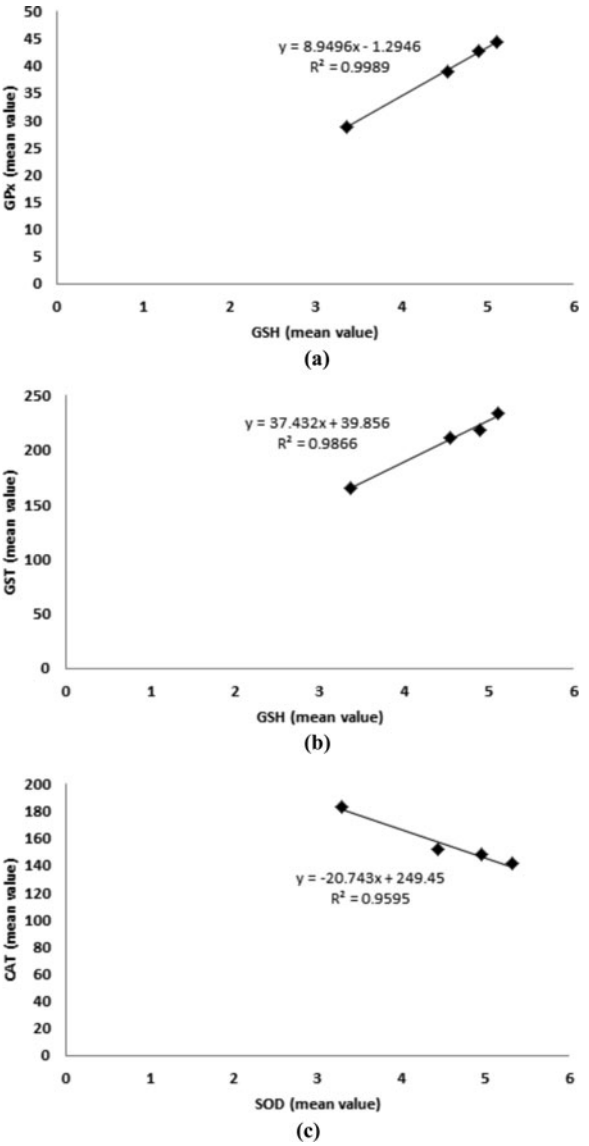

Figure 4. Regression analysis between mean data of (A) reduced glutathione (GSH) and glutathione peroxidase (GPx), (B) reduced glutathione (GSH) and glutathione-S-transferase (GST), and (C) superoxide dismutase (SOD) and catalase (CAT) in fish exposed to $CdCl_2$ at 96 h post exposure.

1989). Cadmium affects calcium balance in rainbow trout (Verbost *et al*. 1987) and induces damage in gill structure of zebra fish, rainbow trout, and tilapia (Karlsson-Norrgren *et al*. 1985; Pratap and Wendelaar Bonga 1993).

The comet assay indicates that Cd exposure of *L. rohita* gill cells elicits DNA damage leading to DNA strand breaks. The finding from our study demonstrates that % tail DNA in gill cells increases with increasing Cd concentration and exposure duration. The highest level of DNA damage observed at the highest Cd concentration after 96 h post exposure. This may be due to continuous exposure to Cd, which leads to the enhancement of ROS formation in gill cells. ROS can react with susceptible biological macromolecules and cause LPO, DNA damage, and protein oxidation (Li *et al*. 2005; Lesser 2006; Cambier *et al*. 2010). Thus, oxidative DNA damage may be the most likely mechanism of genotoxicity of Cd. Moreover, Cd also alters the repair mechanism of DNA by inhibition of an essential DNA mismatch repair (Shi *et al*. 2005). Both the interaction with DNA repair processes and the induction of oxidative DNA damage may account for Cd's genotoxicity (Hartwig 1994).

Lipid peroxidation is acknowledged as being highly deleterious and deriving from attacks by hydroxide free radical, resulting in oxidative damage to organs or tissues (Shi *et al*. 2005; Pandey *et al*. 2008). Hydroxide free radical is produced from H_2O_2 via the Fenton reaction (Evans and Halliwell 1994). CAT is the primary enzyme in scavenging H_2O_2, so when CAT activity is inhibited, more H_2O_2 is available for production of hydroxide free radical. In this study, the decrease of CAT activity in gills of *L. rohita*, together with the increase of SOD activity, could explain the increased MDA level in gills. The elevated MDA level directly reflected that the Cd may cause lipid peroxidation due to production of free radicals. Both the LPO levels and the % Tail DNA increased simultaneously. It is well established that Cd with oxidative stress has the potential to attach on lipids and DNA resulting in clastogenic and molecular damage (Raisuddin and Jha 2004; Jha 2008). Certain fish and non-piscine species (mollusks) have shown a good correlation between level of pollution and extent of genetic damage (Cavas and Konen 2007; Jha 2008). Increase in DNA damage and induction of lipid peroxidation in Cd-treated fish was positively correlated ($r^2 = 0.9952$) at 96 h post exposure.

Superoxide dismutase catalyzes conversion of the superoxide anion radical to molecular oxygen and hydrogen peroxide and this may protect against superoxide-induced oxidative damage (Vutukuru *et al*. 2006). Contaminants such as heavy metals including Cd (Almeida *et al*. 2002; Asagba *et al*. 2008), oils, and phenols (Zhang *et al*. 2005) affect SOD activity in fish (Oruc *et al*. 2004). In the present study, SOD activity in the gills of Cd-treated fish was significantly higher than control fish, possibly in response to toxic effect of superoxide anion radical.

CAT catalyses the conversion of H_2O_2 to water and molecular oxygen and protects biological systems against ROS (Romeo *et al*. 2000). In this study, CAT in Cd-treated groups decreased significantly compared to controls with increased concentration and exposure duration, which indicated that the abilities to protect against H_2O_2 were reduced (Vutukuru *et al*. 2006). The explanation for reduction in CAT activity by Cd exposure may be related to the direct binding of the metal to –SH groups of the enzyme molecule. The reduced CAT activity in gill may also be associated with the compensatory high activity of GPx, which acts as a defense against the formation of H_2O_2 or effective antioxidant responses due to a higher renovation of gill epithelium (Ferreira *et al*. 2005). The negative correlation between increased activity of SOD and decreased activity of CAT suggest that superoxide radicals neutralized by SOD protects the biological system from free radicals while catalase levels

may not be adequate to neutralize hydrogen peroxides, resulting induction of lipid peroxidation.

Glutathione plays an important role in modulation of metal-induced LPO and works as a reducing substrate in oxidative reactions (Schlenk and Rice 1998; Stohs *et al*. 2000). Cadmium exposure caused an increase in GSH content in gill cells of *L. rohita*. Some investigators opined that such Cd-mediated induction of cystolic thiol-rich proteins (GSH and metallothionein) could protect against secondary-induced oxidative stress, probably by maintaining a reduced state in the cell (Tort *et al*. 1996). Transient increase in GSH in our study may be a primary protective response of cells against Cd-induced oxidative stress while increased levels of GSH were probably not sufficient to get rid of oxidative stress, as concomitant increase in LPO amount has been observed in gills of *L. rohita*.

Both GST and GPx are glutathione dependent enzymes and with the assistance of GSH, they play an important role in protecting organs from oxidative stress (Hayes and Strange 1995). GST catalyzes conjugation of many xenobiotics to the tripeptide glutathione while GPx provides optimal protection against oxidative stress (Hermes-Lima 2004) by scavenging H_2O_2 or ROOH, and has been considered to be complementary to CAT (Halliwell and Gutteridge 1999). In the present study, GPx and GST activity increased significantly in gills with increased concentration and exposure duration, which was consistent with most investigations (Atif *et al*. 2005; Bouraoui *et al*. 2008), suggesting that GPx and GST activity could be elevated to resist the Cd-induced toxicity. The positive correlation between GSH and both enzymes (GPx and GST) also shows that increased amount of GSH as well as elevated activity of GPx and GST in Cd-treated fish groups may protect biological systems from oxidative stress.

CONCLUSION

The present study revealed that Cd is responsible for inducing DNA damage, lipid peroxidation, and oxidative stress in *L. rohita* gills. The effect of Cd on DNA damage, lipid peroxidation, non-enzymatic antioxidant, and antioxidant enzymes in gill tissue were concentration and time dependent. The tissue antioxidants seem to counteract the impact of reactive oxygen species while the antioxidant enzymes were unable to prevent DNA damage and lipid peroxidation in the gills due to acute exposure of the metal.

ACKNOWLEDGMENT

The authors are thankful to the Director, National Bureau of Fish Genetic Resources, Lucknow, India, for providing support, suggestions, materials, and a laboratory facility for conducting this study. All experiments were carried out in accordance with the guidelines for the care of experimental animals as approved by the National Bureau of Fish Genetic Resources, Indian Council of Agricultural Research, Canal Ring Road, PO-Dilkusha, Lucknow, India.

REFERENCES

Aebi H. 1984. Catalase *in vitro*. In: Packer L (ed), Methods in Enzymology, pp 121–6. Academic Press, Orlando, FL, USA

Ali D, Nagpure NS, Kumar S, *et al.* 2008. Genotoxicity assessment of acute exposure of chlorpyriphos to fresh water fish *Channa punctatus* (Bloch) using micronucleus assay and alkaline single-cell gel electrophoresis. Chemosphere 71:1823–31

Almeida JA, Diniz YS, Marques,SFG, *et al.* 2002. The use of the oxidative stress responses as biomarkers in Nile tilapia (*Oreochromis niloticus*) exposed to *in vivo* cadmium contamination. Environ Internat 27:673–9

Anderson D, Yu TW, Philips BJ, *et al.* 1994. The effects of various antioxidants and other modifying agents on oxygen radical-generated DNA damage in human lymphocytes in comet assay. Mutat Res 307:261–71

APHA (American Public Health Association). 2005. Standard Methods for the Examination of Water and Wastewater, 21st edit. Washington, DC, USA

Asagba SO, Eriyamremu GE, and Igberaese ME. 2008. Bioaccumulation of cadmium and its biochemical effect on selected tissues of the catfish (*Clarias gariepinus*). Fish Physiol Biochem 34(1):61–9

Atif F, Parvez S, Pandey S, *et al.* 2005. Modulatory effect of cadmium exposure on deltamethrin-induced oxidative stress in *Channa punctata* Bloch. Arch Environ Contam Toxicol 49:371–7

Bechard KM, Gillis PL, and Wood CM. 2008. Trophic transfer of Cd from larval chironomids (*Chironomus riparius*) exposed via sediment or waterborne routes, to zebrafish (Danio rerio): tissue-specific and subcellular comparisons. Aquat Toxicol 90:310–21

Bennett RO and Dooley JK. 1982. Copper uptake by two sympatric species of killifish *Fundulus heteroclitus* (L.) and L. majalis (Walkbaum). J Fish Biol 21:381–98

Beyersmann D and Hechtenberg S. 1997. Cadmium, gene regulation, and cellular signalling in mammalian cells. Toxicol Appl Pharm 144:247–61

Bouraoui Z, Banni M, Ghedira J, *et al.* 2008. Acute effects of cadmium on liver phase I and phase II enzymes and metallothionein accumulation on sea bream *Sparus aurata*. Fish Physiol Biochem 34:201–7

Cambier S, Gonzalez P, Durrieu G, *et al.* 2010. Cadmium-induced genotoxicity in zebrafish at environmentally relevant doses. Ecotoxicology and Environmental Safety 73:312–9

Cavas T and Konen S. 2007. Detection of cytogenetic and DNA damage in peripheral erythrocytes of goldfish (*Carassius auratus*) exposed to a glyphosate formulation using the micronucleus test and the comet assay. Mutagen. 22:263–8

Draper HH, Squires EJ, Mahmooch H, *et al.* 1993. A comparative evaluation of thiobarbituric acid methods for the determination of malondialdehyde in biological materials. Free Radicals Biol Med 15:353–63

Ellman GL. 1959. Tissue sulfhydryl groups. Arch Biochem Biophys 82:70–7

Evans PJ and Halliwell B. 1994. Measurement of iron and copper in biological systems: Bleomycin and copper–phenanthroline assays. Methods in Enzymology 233:82–92

Farag AM, Boese CJ, Woodward DR, *et al.* 1994. Physiological changes and tissue metal accumulation in rainbow trout exposed to foodborne and waterborne metals. Environ Toxicol Chem 13:2021–9

Farombi EO, Adelowo OA, and Ajimoko YR. 2007. Biomarkers of oxidative stress and heavy metal levels as indicators of environmental pollution in African Cat fish (*Clarias gariepinus*) from Nigeria Ogun river. Internat J Environ Res Public Health 4(2):158–65

Ferreira M, Moradas-Ferreira P, and Reis-Henriques MA. 2005. Oxidative stress biomarkers in two resident species, mullet (*Mugil cephalus*) and flounder (*Platichthys flesus*), from a polluted site in River Douro Estuary, Portugal Aquat Toxicol 71:39–48

Flohe L and Gunzler WA. 1984. Assays of glutathione peroxidase. Methods Enzymol 21:105–11

Giaginis C, Gatzidou E, and Theocharis S. 2006. DNA repair systems as targets of cadmium toxicity. Toxicol Appl Pharmacol 213:282–90

Guinee JB, van den Bergh JCJM, Boelens J, *et al.* 1999. Evaluation of risks of metal flows and accumulation in economy and environment. Ecol Econ 30:47–65

Habig W, Pabst M, and Jakoby W. 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem 22:7130–9

Halliwell B and Gutteridge HMC. 1999. Free Radicals in Biology and Medicine. Oxford University Press, Oxford, UK

Hartwig A. 1994. Role of DNA repair inhibition in lead- and cadmium-induced genotoxicity: A review. Environ Health Perspect 102:45–50

Hayes JD and Strange RC. 1995. Potential contribution of the glutathione S-transferase supergene family to resistance to oxidative stress. Free Radical Res 22:193–207

Hermes-Lima M. 2004. Oxygen in biology and biochemistry: Role of free radicals In: Storey KB (ed), Functional Metabolism Regulation and Adaptation, pp 319–68. Wiley-Liss, Hoboken, NJ, USA

Hossain Z and Huq F. 2002. Studies on the interaction between Cd^{2+} ions and nucleobases and nucleotides. J Inorg Biochem 90:97–105

Hutton M. 1983. Sources of cadmium in the environment. Ecotoxicol Environ Saf 7:9–24

Jha AN. 2008. Ecotoxicological applications and significance of the comet assay. Mutagenesis 23:207–21

Jin YH, Clark AB, Slebos RJC, *et al.* 2003. Cadmium is a mutagen that acts by inhibiting mismatch repair. Nat Genet 34:326–9

Kakkar P, Das B, and Viswanathan PN. 1984. A modified spectroscopic assay of superoxide dismutase. Indian J Biochem Biophys 21:130–2

Karlsson-Norrgren L, Runn P, Haux C, *et al.* 1985. Cadmium induced changes in gill morphology of zebrafish, *Brachydynio rerio* (Hamilton-Buchanan), and rainbow trout, *Salmo gairdneri*. J Fish Biol 27:81–95

Klaude M, Eriksson S, Nygren J, *et al.* 1996. The comet assay: Mechanisms and technical considerations. Mutat Res 363:89–96

Kumar R, Nagpure NS, Kushwaha B, *et al.* 2010. Investigation of the genotoxicity of malathion to freshwater teleost fish *Channa punctatus* (Bloch) using the micronucleus test and comet assay. Arch Environ Contam Toxicol 53:617–23

Lesser MP. 2006. Oxidative stress in marine environments: biochemistry and physiological ecology. Ann Rev Physiol 68:253–78

Li XY, Chung IK, Kim JI, *et al.* 2005. Oral exposure to Microcystis increases activity-augmented antioxidant enzymes in the liver of loach (*Misgurnus mizolepis*) and has no effect on lipid peroxidation. Comp Biochem Physiol 141:292–6

Li ZH, Li P, and Randak T. 2011. Evaluating the toxicity of environmental concentrations of waterborne chromium (VI) to a model teleost, oncorhynchus mykiss: A comparative study of *in vivo* and *in vitro*. Comp Biochem Physio 153:402–7

Livingstone DR. 2003. Oxidative stress in aquatic organisms in relation to pollution and aquaculture. Rev Med Vet 154:427–30

Lowry OH, Rosenbrough NM, Farr AL, *et al.* 1951. Protein measurement with Folin phenol reagent. J Biol Chem 193:265–75

Monteiro M, Santos C, Mann Reinier M, *et al.* 2007. Evaluation of cadmium genotoxicity in *Lactuca sativa L.* using nuclear microsatellites. Environ Exp Botany 60:421–7

Nagpure NS, Kumar R, Kushwaha B, *et al.* 2007. Genotoxicity Assessment in Fishes; A Practical Approach. NBFGR, Lucknow, India

Nwani CD, Lakra WS, Nagpure NS, *et al.* 2010. Mutagenic and genotoxic effects of carbosulfan in freshwater fish *Channa punctatus* (Bloch) using micronucleus assay and alkaline single-cell gel electrophoresis. Food Chem Toxicol 48:202–8

OECD (Organization for Economic Cooperation and Development). 1992. Guideline for the Testing of Chemicals: Fish, Acute Toxicity Test. Document 203. Medmenham, UK

Ohkawa H, Ohishi N, and Yagi K. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 8:95–351

Olsson PE, Kling P, and Hogstrand C. 1998. Mechanisms of heavy metal accumulation and toxicity in fish. In: Langston B (eds). Metal Metabolism in Aquatic Environments, pp 321–50. Chapman & Hall Ltd, London, UK

Oruc EO, Sevgiler Y, and Uner N. 2004. Tissue-specific oxidative stress responses in fish exposed to 2,4-D and azinphosmethyl. Comp Biochem Physio 137:43–51

Ozmen I, Bayir A, Cengiz M, *et al.* 2004. Effects of water reuse system on antioxidant enzymes of rainbow trout (*Oncorhynchus mykiss* W., 1792). Veterin Med Czech 49:373–8

Pandey S, Parvez S, Ansari RA, *et al.* 2008. Effects of exposure to multiple trace metals on biochemical, histological and ultrastructural features of gills of a freshwater fish, *Channa punctata* bloch. Chemico-Biological Interactions 174:183–92

Pratap HB and Wendelaar Bonga SE. 1993. Effect of ambient and dietary cadmium on pavement cells, chloride cells and Na^+/K^+-ATPase activity in the gills of the freshwater teleost *Oreochromis mossambicus* at normal and high calcium levels in the ambient water. Aquat Toxicol 26:133–50

Raisuddin S and Jha AN. 2004. Relative sensitivity of fish and mammalian cells to sodium arsenate and arsenite as determined by alkaline single-cell gel electrophoresis and cytokinesis-block micronucleus assay. Environ Mol Mutagen 44:83–9

Risso-de Faverney C, Devaux A, Lafaurie M, *et al.* 2001. Cadmium induces apoptosis and genotoxicity in rainbow trout hepatocytes through generation of reactive oxygene species. Aquat Toxicol 53(1):65–76

Romeo M, Bennani N, Gnassia-Barelli M, *et al.* 2000. Cadmium and copper display different responses towards oxidative stress in the kidney of the sea bass *Dicentrarchus labrax*. Aquat Toxicol 48:185–94

Schlenk D and Rice CD. 1998. Effects of zinc and cadmium treatment on hydrogen peroxide-induced mortality and expression of glutathione and metallothionien in a teleost hepatoma cell line. Aquat Toxicol 43:121–9

Shi H, Sui Y, Wang X, *et al.* 2005. Hydroxyl radical production and oxidative damage induced by cadmium products of membrane constituents chiefly lipids and decreased antioxidant enzymes and reduced glutathione in vitreous. Current Eye Res 19:254–9

Singh NP, McCay MT, Tice RR, *et al.* 1988. A simple technique for quantification of low levels of DNA damage in individual cell. Exp Cell Res 175:184–91

Stohs SJ, Hagchi D, Hassoun E, *et al.* 2000. Oxidative mechanisms in the toxicity of chromium and cadmium ions. J Environ Pathol Toxicol Oncol 19:201–13

Talapatra SN, Ganguly P, Mukhopadhyyay A, *et al.* 2006. Assessment of genetic biomarkers with special reference to micronucleated and binucleated erythrocytes in two fish species grown at Industrial vicinity of thermal power plants, Kolkata, India. Asian J water Environ Poll 4:139–44

Tort L, Krgacin B, Torres P, *et al.* 1996. The effect of cadmium exposure and stress on plasma cortisol, metallothionein levels and oxidative status in rainbow trout (*Oncorhynchus mykiss*) liver. Comp Biochem Physiol 144:29–34

Tsuzuki K, Sugiyama M, and Haramaki N. 1994. DNA-single strand break and cytotoxicity induced by chromate VI, cadmium II and mercury II in hydrogen peroxide-resistant cell lines. Environ Health Perspect 102(3):341–2

Verbost PM, Flick G, Lock RAC, et al. 1987. Cadmium inhibition of Ca^{2+} uptake in rainbow trout gills. Am J Physiol 253:216–21

Verbost PM, Rooij Van J, Flik G, *et al.* 1989. The movement of cadmium through freshwater trout branchial epithelium and its interference with calcium transport. J Exp Biol 145:185–97

Vutukuru SS, Chintada S, Madhavi KR, *et al.* 2006. Acute effects of copper on superoxide dismutase, catalase and lipid peroxidation in the freshwater teleost fish, *Esomus danricus*. Fish Physiol Biochem 32:221–9

Vutukuru SS, Prabhath N Arun, Raghavender M, *et al.* 2007. Effect of Arsenic and Chromium on the Serum Amino-Transferases Activity in Indian Major Carp, *Labeo rohita*. Internat J Environ Res Public Health 4(3):224–7

Waisberg M, Joseph P, Hale B, *et al.* 2003. Molecular and cellular mechanisms of cadmium carcinogenesis. Toxicology 192:95–117

Zhang JF, Liu H, Sun YY, *et al.* 2005. Responses of the antioxidant defenses of the Goldfish *Carassius auratus*, exposed to 2,4-dichlorophenol. Environ Toxicol Pharmacol 19:185–90