Pak. j. sci. ind. res. Ser. B: biol. sci. 2024 **67B**(2) 117-129

Species Specific DNA Damage Induced by Hexavalent Chromium in Three Major Carps

Fariha Latif[a]*, Muhammad Naeem[a], Sana Aziz[b], Rehana Iqbal[a], Safina Kousar[c] and Faiza Ambreen[c]

[a]Institute of Zoology, Bahauddin Zakariya University, Multan, Pakistan
[b]Departement of Zoology, University of Jhang, Jhang, Pakistan
[c]Department of Zoology, Government College Women University, Faisalabad, Pakistan

(received February 26, 2022; revised February 16, 2023; accepted February 24, 2023)

Abstract. Among xenobiotics, metals gained attention because of their toxicity and harmful effects on aquatic ecosystems, fish and human health. Present research work focused on the toxicological effects of hexavalent chromium on the DNA of three major carps *viz*. *Cirrhina mrigala, Labeo rohita* and *Catla catla* by using the comet assay. All three fish species were exposed to four sub-lethal concentrations (2/3rd, 1/3rd, 1/4th and 1/5th of LC_{50}) of chromium for six exposure durations (14, 28, 42, 56, 70 and 84 days). All three fish species exhibited significantly variable exposure dose and time dependent DNA damage in terms of damaged nuclei, GDI and CTL in their erythrocytes. Among the fish species, *C. mrigala* showed maximum DNA damage with the highest damaged nuclei (44.28±25.97%), GDI (1.52±0.84) and CTL (126.91±75.71 µm), whereas significantly lower DNA damage was observed in *C. catla*. At various exposure durations, DNA damage was observed to be significantly higher at 56 days of exposure which then reduced afterward, however, at various exposure concentrations of chromium, maximum DNA damage was observed at 2/3rd of LC_{50} of metal followed by 1/3rd, 1/4th and 1/5th of acute toxicity of chromium for all three fish species under study.

Keywords: major carps, chromium, comet assay, DNA damage

Introduction

Despite the progress in environmental waste management systems, the problems arising due to heavy metal emissions are still posing strong negative effects on aquatic fauna. Especially lithophilic metals are thought to be more dangerous to the environment and essential group of aquatic pollutants because of their persistence, bio-accumulation, bio-magnification and non-biodegradability as they can destroy species diversity. Though aquatic pollution is not a novel phenomenon, the pace of the industrial revolution and urbanization have aggravated its negative impacts on the aquatic environment (Emere and Dibal, 2013). Wastewater is highly polluted with Cr, Ni, Pb, As, Cd and Zn. The pH of wastewater remains slightly acidic, which is harmful to aquatic animals. The level of suspended particles in the wastewater remains greater than the permissible discharge limit (Singh and Mishra, 2021).

Predominant anthropogenic sources of aquatic contamination include mining operations, untreated industrial effluents, domestic sewage, waste dump leachates and combustion emissions (Sultana *et al*., 2016). These sources are adding a variety of pollutants including metallic ions, polycyclic hydrocarbons, dioxins, polychlorinated biphenyls and other xenobiotics in the natural aquatic ecosystems (Valavanidis *et al*., 2006). Metallic ions toxicity affects the physiology and ecology of aquatic organisms due to their specific properties of long persistence, bio-accumulation and bio-magnification in the food chain (Veeraiah *et al*., 2013).

Hexavalent chromium (Cr^{3+}) is considered to be a common environmental pollutant, which widely exists in industrial effluents and wastes (Wang *et al*., 2023). It has been identified as a "Group I human carcinogen" with multisystem and multiorgan toxicity (Zhang *et al*., 2022). Chromium is a stable element but is usually not found in its pure form naturally. Most often, it exists in chromite ore, while trivalent (Cr^{3+}) and hexavalent (Cr^{6+}) forms are stable and predominant. Hexavalent chromium is more toxic than the Cr^{3+} form because of its more permeability across the cell membrane (Singh *et al*., 2022). In aquatic ecosystems, organisms are usually exposed to sub-lethal concentrations of metals for a long duration (Javed, 2013). However, chronic effects of metals on aquatic ecosystems are more severe and

*Author for correspondence; E-mail: farihalatif@bzu.edu.pk

difficult to detect due to less obvious symptoms that take much more time to manifest than those of acute exposures. Metallic ions toxicity caused DNA damage by oxidative stress, competition for ligand binding and molecular mimicry in the cells (Varotto *et al*., 2013). There are multiple assays to evaluate the metals-induced DNA damage, such as detection of sister chromatids exchange, comet assay, quantification of chromosomal aberrations and micro-nucleus test. Most of these are uni-faceted and provide little insight into repair mechanisms or dose-dependent damage. Comet assay is a sensitive and precise assay to quantify and analyze DNA breaks and is widely used to detect genotoxicity caused by various toxic elements in fish (Frenzilli and Lyons, 2013).

Comet is the perfect representation of credible observation and measurement of exposure to toxicants. This technique is used to measure the DNA damage at the single-cell level, also known as single-cell gel electrophoresis. Change in genetic diversity patterns of freshwater fish species exposed to metallic ions contamination has been studied by using comet assay as a field biomarker (Ullah *et al*., 2017). Hence, the current study aimed to evaluate the chronic effects of chromium on the dose and time-dependent DNA damage of three fish species *viz*. *Cirrhina mrigala, Catla catla* and *Labeo rohita*.

Materials and Methods

Acclimatization of test organism. The 120 day old fingerlings of *C. mrigala, C. catla* and *L. rohita* were procured from the fish-rearing earthen ponds of Fisheries Research Farms, University of Agriculture, Faisalabad, Pakistan.

Fingerlings of three fish species were acclimatized to laboratory conditions for 15 days in cemented tanks before acute toxicity tests. During acclimatization, fish were fed to satiation with pelleted feed (30% DP and 3 kcal/g DE) twice daily.

Experimental chemical. Crystalline chromium chloride ($CrCl_2.6H_2O$) was purchased from Merck (Pakistan).

Experimental design. Healthy fish fingerlings of each species with average wet weights, fork and total lengths were selected for the chronic toxicity tests. During chronic exposure of metals, all three fish species were fed the diet to satiation twice a day.

Fish species	Average wet weight (g)	Average fork length (mm)	Average total length (mm)
C. mrigala	14.12±1.92	84.22±1.38	97.68±2.25
C. catla	14.45±1.24	81.25±1.47	84.68±1.45
L. rohita	13.39±1.58	74.53±1.54	80.28±1.86

During chronic exposure of chromium for 84 days, the fish peripheral erythrocytes were collected fortnightly to observe the dose and time-dependent DNA damage in the fish through a comet assay by following the method of Singh *et al*. (1988). *C. catla*, *L. rohita* and *C. mrigala* were exposed to 2/3rd, 1/3rd, 1/4th and 1/5th of their respective 96-h LC_{50} of chromium (Table 1) along with positive control (cyclophosphamide) and negative control (unstressed group). The 96 h LC_{50} values were already calculated in the author's previous research work. The positive control fish were injected intra-peritoneally with 20 µg/g cyclophosphamide in a 4% saline solution. Peripheral blood was collected at 14, 28, 42, 56, 70 and 84 day intervals to analyze DNA damage in the fish erythrocytes exposed to individual metals and MM by using the comet assay.

Comet assay. *Blood sample collection*. After each fortnight (14 days), the blood samples were taken from the caudal vein of the fish through the syringe and first stabilized by using heparin sodium salt and then centrifuged at 1000 rpm for 2 min to separate the erythrocytes (Singh *et al*., 1988)

***Encapsulation*.** Blood samples were diluted with phosphate buffer saline (1 mL) and this mixture(60 µL) was mixed with 110 µL of 1.7% LMP agarose which was evenly coated on the glass slides, that were precoated with 0.5% NMP agarose and covered with glass slip. After 5 min of solidification in the freezer,the

Table 1. Exposure concentrations of chromium to three fish species

Chromium	Treatments (Cr)	*C. mrigala* mg/L	*C. catla* mg/L	*L. rohita* mg/L
	96 H LC_{50}	118.59	93.03	99.78
	2/3rd LC_{50}	79.06	62.02	66.52
	1/3rd LC_{50}	39.53	31.01	33.26
	1/4th LC_{50}	29.65	23.26	24.95
	1/5th LC_{50}	23.72	18.61	19.96

coverslips were removed and evenly coated the slides with 75 µL of LMP agarose (0.8%) and were covered again with glass slips.

***Lysis*.** After the solidification of the gel, the slides without coverslips were immersed in chilled lysing solution (Na_2-EDTA, NaCl, Tris, 1% Triton X and DMSO) and refrigerated for 60 min at 4 °C.

***Alkaline unwinding*.** After lysis, the slides were washed to remove the lysing solution residues and placed in a comet tank (BiocomCS-300V) filled with freshly prepared electrophoresis solution (1mM NaOH, 1mM EDTA) for 20 min to complete the unwinding of DNA strands.

***Electrophoresis*.** After 20 minutes of DNA unwinding, electrophoresis was performed at 300mA and 25V for 25 min in the same solution.

***Neutralization*.** The slides were neutralized by dipping in Tris buffer (0.4M) and stained carefully with ethidium bromide stain.

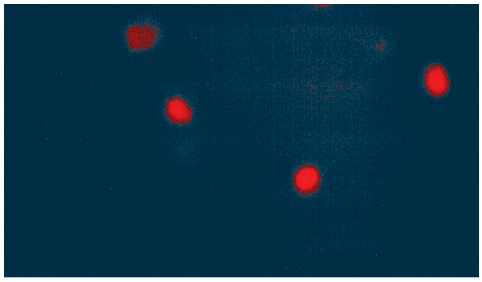
***Slide analysis*.** 150 cells (50/replicate) were examined under an Epi-flourescence microscope with mercury short-arc reflector lamp filters for ethidium bromide at 400X magnification, while using a low lux camera (MD-800, American scope, USA, Fig. 3). The cells with damaged DNA showed a comet-like appearance. DNA damage was estimated by the length of DNA migration in the comet tail.

***Estimation of DNA damage*.** The DNA damage in the peripheral erythrocytes was calculated by visually classifying cells under the following five categories "depending on the tail lengths of comets" as described in Fig. 1.

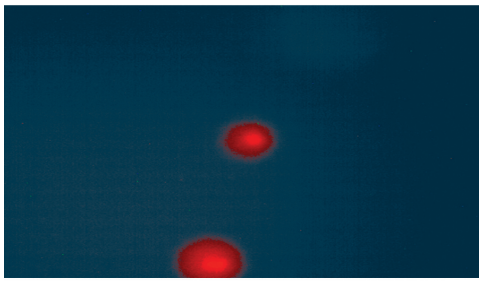
The percentage of damaged nuclei was calculated as Type II + Type III + Type IV, whereas GDI was calculated by using the following formula:

$$GDI = \frac{\text{Type I} + 2\ (\text{Type II}) + 3\ (\text{Type III}) + 4\ (\text{Type IV})}{\text{Type 0} + \text{Type I} + \text{Type II} + \text{Type III} + \text{Type IV}}$$

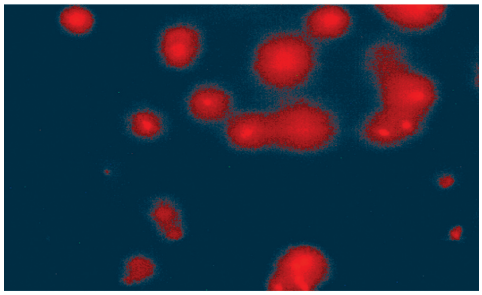
The comet tail length of damaged cells was measured by using TriTekCometScore™ (Summerduck, USA) software (Nassour *et al*., 2016) and CTL (µm) was calculated by adding the comet tail length of all the examined cells. In statistical analyses, the mean values of DNA damage in fish erythrocytes were compared by performing a non-parametric Mann-Whitney U-test. Correlation analyses were also employed to find out the statistical relationships among various parameters.



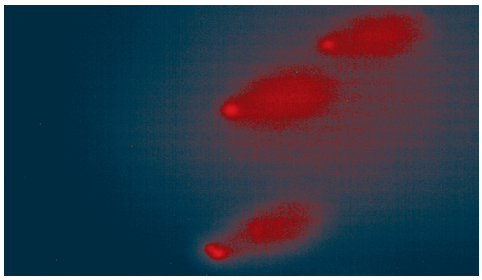
Type 0: Undamaged Nuclei



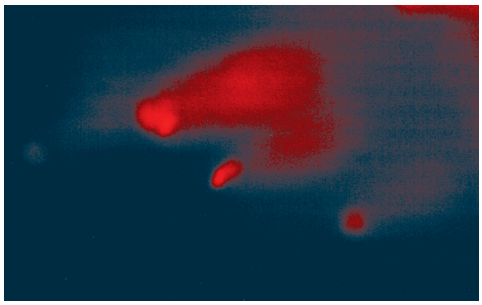
Type I: Low-Level Damaged Nuclei



Type II: Medium Level Damaged Nuclei



Type III: High Level Damaged Nuclei



Type IV: Complete Damaged Nuclei

Fig. 1. Classification of DNA damage induced in the blood erythrocytes of the fish exposed to chromium.

Physico-chemistry of the test media. During each 96-h toxicity trial, water temperature, dissolved oxygen, pH and electrical conductivity were measured through digital meters *viz*. HANNA HI-8424 and HANNI-HI 9146, HANNA HI-99301, respectively. Total hardness, carbon dioxide, sodium, potassium, ammonia, calcium, and magnesium were measured after 24 h by following the methods of A.P.H.A. (2005).

Result and Discussion

DNA damage in *C. mrigala*. Exposure of *C. mirgala* to various concentrations of Cr for 84 days induced significant DNA damage in the blood erythrocytes (Table 2). Among various exposure concentrations of Cr, 2/3rd of LC_{50} caused significantly maximum nuclear damage, GDI and CTL of comets, while this damage was significantly minimum due to negative control significantly higher nuclear damage, GDI and CTL were observed after 56 days of Cr exposure. Cu exposure to *C. mrigala* caused significantly variable induction of GDI and CTL of comets, while the damage to the nuclei was significantly maximum after 56 days, while the same remained significantly minimum due to 14 days of the exposure period.

DNA damage in *Labeo rohita*. The erythrocytes of *L. rohita* showed significantly variable nuclear damage at six exposure concentrations and control that followed the order: positive control ≥ 1/3rd LC_{50}>2/3rdLC_{50}>1/4th LC_{50}>1/5th LC_{50}>negative control (Table 3). The nuclear damage in terms of GDI was significantly maximum at 2/3rd of LC_{50} exposure, followed by 1/3rd LC_{50} with a non-significant difference. However, 2/3rd of LC_{50} exposure of Cr induced longer tail lengths to the comets, while they were short due to negative control. Cd exposure caused significantly higher nuclear damage in terms of GDI after 56 and 42 days of metal exposure. However, the difference between these exposure periods was non-significant.

DNA damage in *Catla catla*. The exposure of *C. catla* to 2/3rd of Cr LC_{50} caused significantly higher damage to the erythrocyte nuclei with the maximum GDI and CTL (Table 4). Peripheral blood erythrocytes of fish exhibited significantly a higher frequency of damaged nuclei after 56 days of exposure as 39.89±25.20%, followed by that of 42 days (39.11±23.52%), while the damage was significantly lower after 84 days of the exposure period (30.67±24.40%). The GDI values were observed to be significantly higher and lower after 42 and 84 days of metal exposure, respectively. Among the three fish species, *C. mrigala* exhibited maximum exposure time dependent percentage damaged nuclei, GDI and CTL followed by *L. rohita* and *C. catla*, whereas the same trend was observed in dose dependent DNA damage among three fish species describe in Fig. 2.

Physico-chemical parameters of test media. Physico-chemical parameters *viz*. temperature, pH, total hardness, dissolved oxygen, electrical conductivity, carbon dioxide,

Table 2. Chromium exposure induced DNA damage in the erythrocytes of *C. mrigala*

	Undamaged nuclei(%)	Damaged nuclei(%)	GDI	CTL (µm)
Dose-dependent				
Negative control	94.89±0.34^{a}	1.33±0.59^{f}	0.06±0.01^{f}	3.44±0.04^{f}
Positive control	30.44±2.84^{c}	53.33±2.79^{c}	1.79±0.07^{c}	134.67±2.94^{d}
2/3rd of LC_{50}	11.67±2.75^{f}	73.44±8.08^{a}	2.43±0.21^{a}	217.21±22.08^{a}
1/3rd of LC_{50}	21.11±5.69^{e}	61.33±6.92^{b}	2.06±0.22^{b}	179.52±29.25^{b}
1/4th of LC_{50}	26.89±4.98^{d}	48.78±10.69^{d}	1.66±0.27^{d}	145.45±34.53^{c}
1/5th of LC_{50}	35.00±6.81^{b}	27.44±10.67^{e}	1.14±0.18^{e}	81.18±25.04^{e}
Time-dependent				
14 days	38.11±29.16^{a}	36.33±23.99^{d}	1.33±0.73^{e}	10.65±64.93^{e}
28 days	36.11±30.03^{bc}	42.33±27.75^{c}	1.50±0.87^{c}	127.26±80.90^{c}
42 days	37.22±30.98^{ab}	46.22±29.63^{b}	1.60±0.96^{b}	135.60±86.85^{b}
56 days	34.89±30.64^{c}	52.11±27.38^{a}	1.68±0.88^{a}	149.73±84.91^{a}
70 days	36.89±29.47^{ab}	46.44±26.05^{b}	1.56±0.84^{bc}	132.85±74.13^{b}
84 days	36.78±32.22^{ab}	42.22±26.72^{c}	1.47±0.87^{d}	115.38±77.53^{d}

The means sharing same letters in the column are statistically at par (P<0.05).

sodium, potassium, ammonia, calcium and magnesium were measured and recorded during the research work and presented in Table 5.

The negative effects of heavy metals are not limited to acute and chronic toxicity exposures rather they get accumulated in various fish tissues and cause genotoxicity as well (Kehinde *et al*., 2016). Heavy metals are capable of causing genotoxicity in the fish either by direct damage of DNA or indirectly through oxidative stress/damage, inhibiting DNA repair mechanisms and interacting with tumor suppressor proteins (Bolognesi and Cirillo, 2014). The genotoxic effects of metals can be monitored by using various biomarker assays but during the present study, cometthe assay was used to detect DNA damage in fish peripheral erythrocytes. The comet test is considered a reliable and sensitive method to measure low-level DNA damage even 0.1 DNA break/10^9 Da in the nuclei (Ali *et al*., 2008). During our research, chromium exposure caused dose and time dependent DNA damage in the erythrocytes of all three major carps. Chronic exposure to Cr^{6+}

Table 3. Chromium exposure induced DNA damage in the erythrocytes of *L. rohita*

	Undamaged nuclei(%)	Damaged nuclei(%)	GDI	CTL (µm)
Dose-dependent				
Negative control	95.11±0.54^{a}	1.11±0.54^{e}	0.06±0.00^{e}	3.42±0.03^{f}
Positive control	28.22±5.60^{c}	55.44±3.85^{a}	1.84±0.12^{b}	136.93±4.38^{c}
2/3rd of LC_{50}	27.44±5.47^{cd}	53.78±8.16^{b}	1.92±0.23^{a}	165.86±25.61^{a}
1/3rd of LC_{50}	19.22±6.64^{e}	54.00±9.38^{a}	1.91±0.23^{a}	162.65±34.06^{b}
1/4th of LC_{50}	25.89±6.53^{d}	41.89±10.54^{c}	1.55±0.24^{c}	130.89±36.04^{d}
1/5th of LC_{50}	35.78±11.54^{b}	22.22±8.47^{d}	1.02±0.23^{d}	81.51±22.18^{e}
Time-dependent				
14 days	35.11±30.06^{d}	35.00±22.69^{c}	1.37±0.73^{c}	98.50±57.81^{f}
28 days	38.78±28.52^{c}	37.00±24.13^{b}	1.37±0.75^{c}	107.55±60.40^{d}
42 days	36.56±29.33^{d}	43.11±24.66^{a}	1.50±0.82^{b}	125.82±74.98^{b}
56 days	34.78±30.96^{d}	44.22±23.69^{a}	1.56±0.80^{a}	133.28±71.84^{a}
70 days	41.56±27.20^{b}	37.00±21.31^{b}	1.30±0.69^{d}	114.36±63.88^{c}
84 days	44.89±28.11^{a}	32.11±22.93^{d}	1.20±0.76^{e}	101.76±67.28^{e}

The means sharing same letters in the column are statistically at par (P<0.05).

Table 4. Chromium exposure induced DNA damage in the erythrocytes of *C. catla*

	Undamaged nuclei (%)	Damaged nuclei(%)	GDI	CTL (µm)
Dose-dependent				
Negative control	96.00±1.03^{a}	1.00±0.69^{f}	0.05±0.01^{f}	3.45±0.02^{f}
Positive control	25.22±5.63^{de}	54.33±2.29^{b}	1.88±0.08^{b}	138.42±4.53^{c}
2/3rd of LC_{50}	23.44±6.71^{e}	57.33±7.41^{a}	1.96±0.19^{a}	162.04±28.06^{a}
1/3rd of LC_{50}	27.33±3.45^{d}	48.44±8.85^{c}	1.68±0.22^{c}	139.68±21.37^{b}
1/4th of LC_{50}	30.78±9.07^{c}	28.00±6.42^{d}	1.24±0.24^{d}	89.23±20.61^{d}
1/5th of LC_{50}	38.11±8.80^{b}	18.22±5.97^{e}	0.96±0.13^{e}	64.07±22.65^{e}
Time-dependent				
14 days	35.89±29.64^{d}	28.23±21.37^{c}	1.24±0.68^{c}	85.64±59.30^{d}
28 days	43.67±26.58^{a}	35.22±22.18^{b}	1.29±0.72^{c}	98.35±53.61^{c}
42 days	37.22±29.75^{cd}	39.11±23.52^{a}	1.46±0.77^{a}	112.26±63.78^{b}
56 days	40.11±28.13^{b}	39.89±25.20^{a}	1.37±0.79^{b}	116.12±70.98^{a}
70 days	39.44±28.93^{bc}	33.33±22.77^{b}	1.28±0.73^{c}	100.42±61.90^{c}
84 days	44.56±29.97^{a}	30.67±24.41^{c}	1.14±0.77^{d}	84.09±60.69^{d}

Means sharing same letters in the column are statistically at par (P<0.05).

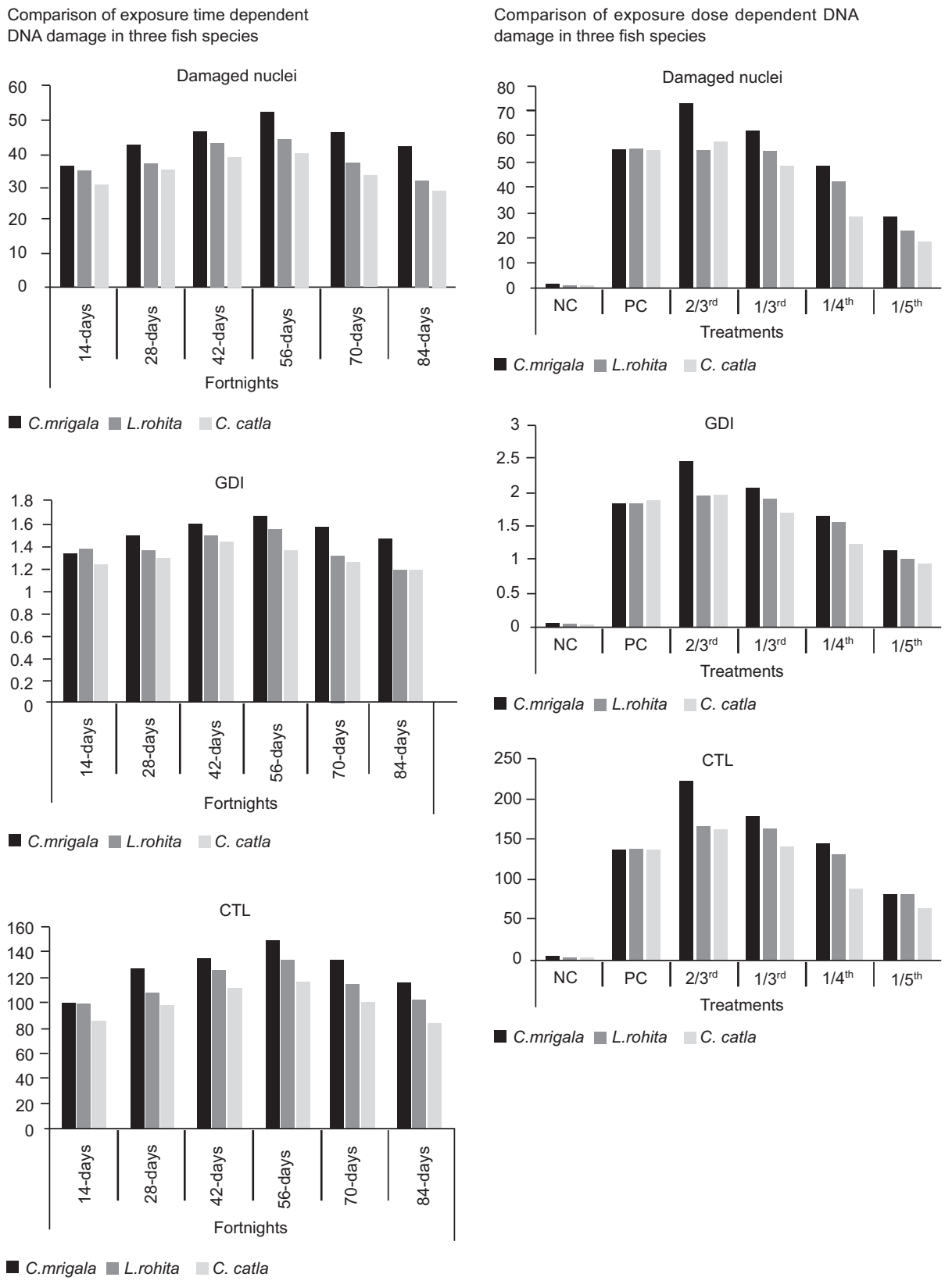


Fig. 2. Comparison of fish species for dose and time dependent DNA damage induced by chromium exposure.

Table 5. Physico-chemical parameters of chromium exposed test media for three fish species

Concentration	Fish species	Temperature (°C)	pH	Total hardness (mg/L)	Dissolved Oxygen (mg/L)	Electrical conductivity (mS/cm)	Carbon dioxide (mg/L)	Sodium (mg/L)	Potassium (mg/L)	Ammonia (mg/L)	Calcium (mg/L)	Magnesium (mg/L)
	C. mirgala	30.02±0.01	7.51±0.03	250.01±0.02	4.61±0.17	2.60±0.14	1.73±0.11	302.08±3.67	8.52±0.27	1.21±0.02	22.72±0.09	48.31±0.17
$2/3^{rd}$ LC_{50}	*L. rohita*	29.99±0.01	7.49±0.01	250.01±0.01	4.59±0.12	2.60±0.14	1.75±0.12	301.88±2.98	8.73±0.73	1.18±0.01	23.11±0.10	48.06±0.17
	C. catla	30.00±0.01	7.50±0.02	250.02±0.03	4.59±0.13	2.50±0.09	1.81±0.13	300.93±1.99	8.78±0.83	1.12±0.01	23.30±0.18	47.94±0.13
	C. mirgala	30.46±0.03	7.51±0.02	250.00±0.01	4.89±0.23	2.87±0.23	1.85±0.14	302.50±4.19	8.70±0.65	1.24±0.03	23.36±0.23	47.90±0.09
$1/3^{rd}$ LC_{50}	*L. rohita*	30.06±0.02	7.48±0.01	250.01±0.02	4.77±0.20	2.84±0.16	1.73±0.11	302.07±3.67	8.90±1.05	1.25±0.04	22.60±0.05	48.38±0.21
	C. catla	30.05±0.02	7.49±0.01	250.01±0.02	4.30±0.08	2.44±0.07	1.63±0.09	300.40±1.19	8.09±0.05	1.22±0.02	23.60±0.30	47.75±0.07
	C. mirgala	30.26±0.03	7.50±0.02	251.00±0.03	5.48±0.30	3.07±0.25	1.52±0.06	302.23±3.97	8.80±1.03	1.26±0.05	23.38±0.26	47.90±0.10
$1/4^{th}LC_{50}$	*L. rohita*	29.93±0.01	7.49±0.01	250.09±0.04	5.31±0.27	2.85±0.17	1.57±0.07	301.97±3.45	8.68±0.53	1.27±0.06	22.61±0.07	48.42±0.23
	C. catla	30.41±0.03	7.50±0.02	250.02±0.03	5.12±0.25	2.96±0.23	1.60±0.08	301.48±2.16	8.39±0.13	1.23±0.02	23.61±0.30	47.75±0.07
	C. mirgala	30.03±0.01	7.48±0.01	250.01±0.02	5.64±0.31	3.05±0.20	1.30±0.04	301.95±3.13	8.95±1.10	1.25±0.04	23.17±0.12	48.02±0.15
$1/5^{th}LC_{50}$	*L. rohita*	30.01±0.02	7.51±0.04	250.08±0.03	5.67±0.33	2.86±0.19	1.23±0.03	300.85±1.55	8.56±0.39	1.26±0.05	22.53±0.02	48.47±0.25
	C. catla	30.01±0.01	7.50±0.01	250.01±0.02	5.78±0.36	3.08±0.30	1.30±0.04	302.21±3.84	8.61±0.47	1.24±0.03	22.61±0.07	48.37±0.19

DNA damage induced by chromium exposure in the erythrocytes of *C. mrigala*

56 days	Negative control	94.67 ± 1.15^{a}	3.33 ± 1.15^{d}	2.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	2.00 ± 0.00^{f}	0.07 ± 0.01^{e}	3.39 ± 0.04^{f}
	Positive control	32.67 ± 1.15^{b}	14.67 ± 4.16^{b}	24.67 ± 3.06^{a}	10.67 ± 1.15^{c}	17.33 ± 0.31^{b}	52.67 ± 4.62^{d}	1.65 ± 0.09^{c}	133.72 ± 0.82^{d}
	$2/3^{rd}$ LC_{50}	13.33 ± 3.06^{d}	8.67 ± 3.06^{c}	19.33 ± 3.06^{c}	26.67 ± 3.06^{ab}	32.00 ± 2.00^{a}	78.00 ± 2.00^{a}	2.55 ± 0.08^{a}	237.67 ± 0.22^{a}
	$1/3^{rd}$ LC_{50}	14.67 ± 1.35^{d}	14.00 ± 4.00^{b}	22.67 ± 3.06^{ab}	30.00 ± 2.00^{a}	18.67 ± 4.16^{b}	71.33 ± 4.16^{b}	2.24 ± 0.13^{b}	209.87 ± 0.84^{b}
	$1/4^{th}$ LC_{50}	19.33 ± 1.15^{c}	16.67 ± 1.15^{ab}	18.67 ± 3.06^{c}	26.00 ± 2.00^{b}	19.33 ± 1.15^{b}	64.00 ± 2.00^{c}	2.09 ± 0.03^{b}	195.13 ± 0.97^{c}
	$1/5^{th}$ LC_{50}	34.67 ± 1.24^{b}	20.67 ± 3.06^{a}	20.67 ± 3.06^{bc}	14.00 ± 2.00^{c}	10.00 ± 2.00^{c}	44.67 ± 4.16^{e}	1.44 ± 0.07^{d}	118.59 ± 1.12^{e}
70 days	Negative control	94.67 ± 2.31^{a}	4.67 ± 1.15^{cd}	0.67 ± 1.15^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.67 ± 1.15^{e}	0.06 ± 0.03^{e}	3.48 ± 0.09^{f}
	Positive control	30.00 ± 3.46^{c}	16.67 ± 3.06^{b}	20.67 ± 3.06^{ab}	12.00 ± 2.00^{c}	21.33 ± 3.06^{b}	54.00 ± 7.21^{c}	1.78 ± 0.18^{c}	139.11 ± 1.42^{d}
	$2/3^{rd}$ LC_{50}	11.33 ± 3.06^{e}	14.00 ± 2.00^{c}	22.67 ± 2.31^{a}	17.33 ± 3.06^{b}	34.67 ± 1.15^{a}	74.67 ± 4.62^{a}	2.50 ± 0.12^{a}	217.12 ± 0.47^{a}
	$1/3^{rd}$ LC_{50}	22.00 ± 2.00^{d}	16.00 ± 2.00^{bc}	17.33 ± 3.06^{bc}	23.33 ± 3.06^{a}	21.33 ± 3.06^{b}	62.00 ± 4.00^{b}	2.06 ± 0.07^{b}	172.89 ± 0.71^{b}
	$1/4^{th}$ LC_{50}	28.00 ± 2.00^{c}	18.67 ± 3.06^{b}	20.00 ± 2.00^{ab}	20.67 ± 4.16^{ab}	12.67 ± 3.06^{c}	53.33 ± 4.62^{c}	1.71 ± 0.12^{c}	163.84 ± 0.50^{c}
	$1/5^{th}$ LC_{50}	35.33 ± 1.15^{b}	30.67 ± 4.16^{a}	14.67 ± 4.16^{c}	10.67 ± 2.31^{c}	8.67 ± 1.15^{c}	34.00 ± 4.00^{d}	1.27 ± 0.04^{d}	100.69 ± 1.27^{e}
84 days	Negative control	95.33 ± 3.06^{a}	3.33 ± 3.06^{e}	1.33 ± 1.15^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	1.33 ± 1.15^{e}	0.06 ± 0.03^{f}	3.45 ± 0.03^{f}
	Positive control	31.33 ± 3.06^{bc}	13.33 ± 3.06^{d}	20.67 ± 2.13^{a}	13.33 ± 3.06^{b}	21.33 ± 3.06^{a}	55.33 ± 3.06^{b}	1.80 ± 0.09^{c}	136.25 ± 1.97^{c}

continued.....

	$2/3^{rd}$ LC_{50}	16.00 ± 3.46^{e}	18.00 ± 2.00^{cd}	17.33 ± 1.15^{ab}	24.00 ± 2.00^{a}	24.67 ± 4.16^{a}	66.00 ± 4.00^{a}	2.23 ± 0.14^{a}	204.67 ± 1.46^{a}
	$1/3^{rd}$ LC_{50}	20.00 ± 5.29^{de}	22.67 ± 5.03^{bc}	11.03 ± 1.15^{c}	26.67 ± 3.06^{a}	19.33 ± 3.06^{ab}	57.33 ± 6.11^{b}	2.03 ± 0.19^{b}	155.36 ± 0.95^{b}
	$1/4^{th}$ LC_{50}	24.67 ± 2.31^{cd}	29.33 ± 3.06^{b}	20.67 ± 3.06^{a}	14.00 ± 2.00^{b}	11.33 ± 3.06^{b}	46.00 ± 2.00^{c}	1.58 ± 0.05^{d}	117.99 ± 1.09^{d}
	$1/5^{th}$ LC_{50}	33.33 ± 5.03^{b}	39.33 ± 7.02^{a}	12.67 ± 4.16^{bc}	8.67 ± 2.31^{c}	6.00 ± 2.00^{c}	27.33 ± 2.31^{d}	1.15 ± 0.08^{e}	75.53 ± 0.35^{e}
DNA damage induced by chromium exposure in the erythrocytes of *L. rohita*									
14 days	Negative control	94.67 ± 1.15^{a}	4.67 ± 1.15^{f}	0.67 ± 1.11^{d}	0.00 ± 0.00^{c}	0.00 ± 0.00^{f}	0.67 ± 1.15^{f}	0.06 ± 0.02^{e}	3.41 ± 0.02^{f}
	Positive control	30.67 ± 2.31^{b}	10.00 ± 3.46^{e}	24.68 ± 4.16^{a}	15.33 ± 3.06^{a}	19.33 ± 3.06^{b}	59.33 ± 3.06^{a}	1.83 ± 0.03^{b}	137.54 ± 3.92^{b}
	$2/3^{rd}$ LC_{50}	20.00 ± 2.00^{c}	23.33 ± 3.06^{d}	11.33 ± 2.31^{b}	18.00 ± 2.00^{a}	27.33 ± 3.06^{a}	56.67 ± 1.15^{b}	2.09 ± 0.05^{a}	167.66 ± 0.76^{a}
	$1/3^{rd}$ LC_{50}	14.00 ± 2.00^{d}	42.67 ± 2.31^{b}	10.67 ± 3.06^{bc}	17.33 ± 2.21^{a}	15.33 ± 2.31^{c}	43.33 ± 1.15^{c}	1.17 ± 0.05^{d}	122.21 ± 0.67^{c}
	$1/4^{th}$ LC_{50}	32.67 ± 1.15^{b}	36.67 ± 5.03^{c}	10.00 ± 2.00^{bc}	10.67 ± 1.15^{b}	10.00 ± 2.00^{d}	30.67 ± 4.16^{d}	1.29 ± 0.08^{c}	87.78 ± 0.43^{d}
	$1/5^{th}$ LC_{50}	18.67 ± 2.31^{c}	62.00 ± 2.00^{a}	6.67 ± 1.15^{cd}	8.67 ± 3.06^{b}	4.00 ± 2.00^{e}	19.33 ± 3.06^{e}	1.17 ± 0.10^{d}	72.42 ± 0.27^{e}
28 days	Negative control	95.33 ± 1.15^{a}	3.33 ± 1.15^{d}	1.33 ± 1.13^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	1.33 ± 1.15^{e}	0.06 ± 0.02^{d}	3.39 ± 0.05^{f}
	Positive control	27.33 ± 2.31^{c}	11.33 ± 4.16^{c}	21.33 ± 3.06^{a}	14.00 ± 2.00^{b}	26.00 ± 2.00^{a}	61.33 ± 6.43^{a}	2.00 ± 0.14^{a}	141.62 ± 2.61^{b}
	$2/3^{rd}$ LC_{50}	28.67 ± 4.62^{c}	12.67 ± 3.06^{c}	21.33 ± 3.06^{a}	13.33 ± 3.07^{b}	24.00 ± 2.00^{a}	58.67 ± 7.57^{a}	1.91 ± 0.19^{a}	168.84 ± 0.83^{a}
	$1/3^{rd}$ LC_{50}	26.67 ± 2.31^{c}	26.00 ± 2.00^{b}	12.00 ± 2.00^{b}	20.00 ± 2.00^{a}	15.33 ± 4.16^{b}	47.33 ± 1.15^{b}	1.71 ± 0.07^{b}	137.40 ± 0.49^{c}
	$1/4^{th}$ LC_{50}	16.67 ± 3.06^{d}	45.33 ± 3.06^{a}	9.33 ± 1.15^{b}	17.33 ± 1.15^{a}	11.33 ± 2.31^{b}	38.00 ± 0.00^{c}	1.61 ± 0.06^{b}	123.12 ± 0.51^{d}
	$1/5^{th}$ LC_{50}	38.00 ± 4.00^{b}	46.67 ± 3.06^{a}	4.67 ± 1.15^{c}	8.00 ± 2.00^{c}	2.67 ± 1.15^{c}	15.33 ± 1.15^{d}	0.91 ± 0.04^{c}	70.90 ± 0.83^{e}
42 days	Negative control	94.67 ± 1.15^{a}	4.67 ± 1.15^{d}	0.67 ± 1.15^{d}	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.67 ± 1.15^{d}	0.06 ± 0.02^{e}	3.42 ± 0.03^{f}
	Positive control	30.67 ± 3.06^{bc}	16.67 ± 3.06^{c}	20.00 ± 2.00^{ab}	12.67 ± 2.31^{c}	20.00 ± 2.00^{b}	52.67 ± 1.15^{b}	1.75 ± 0.03^{c}	134.27 ± 1.58^{c}
	$2/3^{rd}$ LC_{50}	21.33 ± 1.15^{d}	12.67 ± 3.06^{c}	14.00 ± 3.46^{c}	19.33 ± 3.06^{b}	32.67 ± 2.31^{a}	66.00 ± 3.46^{a}	2.29 ± 0.096^{a}	211.48 ± 1.12^{a}
	$1/3^{rd}$ LC_{50}	14.00 ± 2.00^{e}	24.00 ± 4.00^{b}	23.33 ± 3.06^{a}	16.00 ± 2.00^{bc}	22.67 ± 1.15^{b}	62.00 ± 2.00^{a}	2.09 ± 0.03^{b}	91.45 ± 1.03^{b}
	$1/4^{th}$ LC_{50}	24.67 ± 4.16^{cd}	26.00 ± 2.00^{b}	13.33 ± 4.16^{c}	24.00 ± 2.00^{a}	12.00 ± 2.00^{c}	49.33 ± 3.06^{b}	1.73 ± 0.12^{c}	126.11 ± 0.83^{d}
	$1/5^{th}$ LC_{50}	34.00 ± 5.29^{b}	38.00 ± 2.00^{a}	17.33 ± 3.06^{cd}	7.33 ± 1.15^{d}	3.33 ± 1.15^{d}	28.00 ± 3.46^{c}	1.08 ± 0.09^{d}	88.20 ± 0.63^{e}
56 days	Negative control	96.00 ± 2.00^{a}	3.33 ± 1.15^{e}	0.67 ± 1.15^{d}	0.00 ± 0.00^{e}	0.00 ± 0.00^{c}	0.67 ± 1.15^{e}	0.05 ± 0.03^{e}	3.45 ± 0.03^{f}
	Positive control	17.33 ± 4.62^{cd}	29.33 ± 4.16^{b}	13.33 ± 4.16^{c}	18.00 ± 2.00^{bc}	22.00 ± 2.00^{a}	53.33 ± 1.15^{c}	1.98 ± 0.08^{b}	131.14 ± 2.17^{d}
	$2/3^{rd}$ LC_{50}	33.33 ± 3.06^{b}	16.67 ± 3.06^{d}	10.67 ± 3.06^{c}	20.00 ± 2.00^{b}	19.33 ± 3.06^{ab}	50.00 ± 0.00^{c}	1.75 ± 0.03^{c}	146.75 ± 1.75^{c}
	$1/3^{rd}$ LC_{50}	12.67 ± 1.15^{d}	20.00 ± 2.00^{cd}	14.00 ± 2.00^{bc}	33.33 ± 1.15^{a}	20.00 ± 2.00^{a}	67.33 ± 2.31^{a}	2.28 ± 0.05^{a}	211.46 ± 1.13^{a}
	$1/4^{th}$ LC_{50}	20.00 ± 2.00^{c}	22.00 ± 2.00^{c}	22.67 ± 2.31^{a}	15.33 ± 2.31^{c}	20.00 ± 2.00^{a}	58.00 ± 2.00^{b}	1.93 ± 0.06^{b}	183.77 ± 0.38^{b}
	$1/5^{th}$ LC_{50}	29.33 ± 3.06^{b}	34.67 ± 1.15^{a}	17.33 ± 1.15^{b}	10.00 ± 2.00^{d}	8.67 ± 1.15^{b}	36.00 ± 3.46^{d}	1.34 ± 0.11^{d}	123.13 ± 0.50^{e}
70 days	Negative control	95.33 ± 1.15^{a}	3.33 ± 1.15^{e}	1.33 ± 1.15^{c}	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}	1.33 ± 1.15^{f}	0.06 ± 0.02^{e}	3.39 ± 0.10^{f}
	Positive control	30.67 ± 2.31^{c}	16.67 ± 4.16^{d}	19.33 ± 2.31^{a}	14.00 ± 2.00^{a}	19.33 ± 3.06^{b}	52.67 ± 6.43^{b}	1.75 ± 0.16^{b}	142.26 ± 2.68^{c}
	$2/3^{rd}$ LC_{50}	30.67 ± 3.06^{c}	22.00 ± 2.00^{c}	10.67 ± 3.06^{b}	13.33 ± 1.15^{ab}	23.33 ± 2.31^{a}	47.33 ± 3.06^{c}	1.77 ± 0.08^{b}	137.31 ± 0.33^{d}
	$1/3^{rd}$ LC_{50}	20.67 ± 3.06^{d}	23.33 ± 3.06^{c}	22.67 ± 2.31^{a}	14.67 ± 3.06^{a}	18.67 ± 1.15^{b}	56.00 ± 0.00^{a}	1.87 ± 0.03^{a}	167.77 ± 0.94^{a}

continued.....

	1/4th LC_{50}	30.00±2.00^{c}	26.67±4.16^{b}	24.00±5.29^{a}	12.00±2.00^{b}	7.33±1.15^{c}	43.33±6.11^{d}	1.40±0.09^{c}	161.91±0.57^{b}
	1/5th LC_{50}	42.00±2.00^{b}	36.67±1.15^{a}	9.33±1.15^{b}	7.33±3.06^{c}	4.67±1.15^{d}	21.33±3.06^{e}	0.96±0.10^{d}	73.53±0.35^{e}
84 days	Negative control	94.67±1.15^{a}	3.33±1.15^{d}	2.00±0.00^{d}	0.00±0.00^{e}	0.00±0.00^{e}	2.00±0.00^{e}	0.07±0.01^{d}	3.48±0.03^{f}
	Positive control	32.67±2.31^{c}	14.00±2.00^{c}	20.67±2.31^{a}	13.33±2.31^{b}	19.33±3.06^{b}	53.33±3.06^{a}	1.73±0.09^{a}	134.74±1.34^{c}
	2/3rd LC_{50}	30.67±1.15^{c}	25.33±1.15^{b}	9.33±1.15^{c}	14.00±2.00^{b}	20.67±3.23^{a}	44.00±2.00^{b}	1.69±0.08^{a}	163.14±1.29^{a}
	1/3rd LC_{50}	27.33±3.06^{d}	24.67±3.06^{b}	12.00±2.00^{b}	22.67±1.15^{a}	13.33±3.06^{c}	48.00±2.00^{b}	1.70±0.11^{a}	145.62±0.22^{b}
	1/4th LC_{50}	31.33±4.16^{c}	36.67±4.62^{a}	10.67±1.15^{bc}	7.33±2.31^{c}	14.00±2.00^{c}	32.00±5.29^{c}	1.36±0.14^{b}	102.65±1.15^{d}
	1/5th LC_{50}	52.67±3.06^{b}	34.00±3.46^{a}	8.67±1.15^{c}	3.33±1.15^{d}	1.33±1.15^{d}	13.33±1.15^{d}	0.67±0.03^{c}	60.90±0.83^{e}
DNA damage induced by chromium exposure in the erythrocytes of *C. catla*									
14 days	Negative control	95.33±1.15^{a}	3.33±1.15 e	1.33±1.15^{d}	0.00±0.00^{d}	0.00±0.00^{e}	1.33±1.15^{f}	0.06±0.02^{d}	3.46±0.08^{e}
	Positive control	14.67±3.06^{c}	30.67±4.62^{c}	21.33±6.11^{a}	12.67±3.06^{a}	20.67±1.15^{b}	54.67±5.03^{a}	1.94±0.07^{a}	140.74±0.49^{a}
	2/3rd LC_{50}	28.00±6.00^{b}	20.67±6.11^{d}	15.33±3.06^{b}	10.67±1.15^{ab}	25.33±4.16^{a}	51.33±1.15^{b}	1.85±0.13^{a}	141.94±2.14^{a}
	1/3rd LC_{50}	30.67±4.16^{b}	30.00±2.00^{c}	19.33±3.06^{ab}	10.00±2.00^{ab}	10.00±2.00^{c}	39.33±6.11^{c}	1.39±0.15^{b}	123.99±1.19^{b}
	1/4th LC_{50}	22.00±6.00^{bc}	54.00±4.00^{b}	9.33±3.06^{c}	8.67±1.15^{b}	6.00±2.00^{d}	24.00±2.00^{d}	1.23±0.10^{b}	72.68±1.86^{c}
	1/5th LC_{50}	24.67±4.16^{b}	62.00±3.46^{a}	5.33±1.15^{cd}	5.33±1.15^{c}	2.67±1.15^{de}	13.33±1.15^{e}	0.99±0.08^{c}	31.02±1.04^{d}
28 days	Negative control	96.67±1.15^{a}	2.67±1.15^{e}	0.67±1.15^{d}	0.00±0.00^{e}	0.00±0.00^{d}	0.67±1.15^{e}	0.04±0.02^{e}	3.48±0.10^{f}
	Positive control	27.33±4.16^{d}	15.33±2.31^{d}	20.67±4.16^{a}	14.00±2.00^{c}	22.67±2.31^{a}	57.33±4.16^{a}	1.89±0.11^{a}	145.86±3.23^{a}
	2/3rd LC_{50}	29.33±6.43^{d}	14.67±1.15^{d}	11.33±3.06^{b}	19.33±2.31^{b}	25.33±3.06^{a}	56.00±6.93^{a}	1.97±0.20^{a}	137.84±1.57^{b}
	1/3rd LC_{50}	30.67±3.06^{d}	24.67±1.15^{c}	10.67±3.06^{b}	22.67±1.15^{a}	11.33±1.15^{b}	44.67±3.06^{b}	1.59±0.06^{b}	128.83±0.83^{c}
	1/4th LC_{50}	34.67±32.00^{c}	32.00±4.00^{b}	6.67±1.15^{c}	18.67±1.15^{b}	8.00±2.00^{bc}	33.33±2.31^{c}	1.33±0.11^{c}	100.69±1.27^{d}
	1/5th LC_{50}	43.33±4.16^{b}	37.33±2.31^{a}	8.67±3.06^{bc}	4.67±1.15^{d}	6.00±2.00^{c}	19.33±3.06^{d}	0.93±0.08^{d}	73.42±0.27^{e}
42 days	Negative control	94.67±1.15^{a}	3.33±1.15^{e}	2.00±0.00^{c}	0.00±0.00^{e}	0.00±0.00^{e}	2.00±0.00^{e}	0.07±0.01^{f}	3.41±0.08^{f}
	Positive control	28.67±3.06^{c}	17.33±4.26^{d}	9.33±1.15^{b}	18.67±3.06^{a}	26.00±2.00^{a}	54.00±2.00^{b}	1.96±0.05^{b}	133.80±1.61^{c}
	2/3rd LC_{50}	13.33±2.31^{d}	23.33±3.06^{c}	23.33±3.06^{a}	16.67±2.31^{ab}	23.33±1.15^{ab}	63.33±1.15^{a}	2.13±0.03^{a}	179.54±1.10^{a}
	1/3rd LC_{50}	28.67±3.06^{c}	15.33±2.31^{d}	20.67±4.16^{a}	14.00±2.00^{bc}	21.33±2.31^{b}	56.00±2.00^{b}	1.84±0.02^{c}	157.71±1.25^{b}
	1/4th LC_{50}	17.33±3.06^{d}	45.33±3.06^{a}	6.67±2.31^{b}	13.33±2.31^{c}	17.33±1.15^{c}	37.33±1.15^{c}	1.68±0.07^{d}	123.13±0.50^{d}
	1/5th LC_{50}	40.67±3.06^{b}	37.33±3.06^{b}	5.33±2.31^{bc}	8.67±1.15^{d}	8.00±2.00^{d}	22.00±4.00^{d}	1.06±0.11^{e}	75.98±0.70^{e}
56 days	Negative control	95.33±1.15^{a}	3.33±1.15^{e}	1.33±1.15^{c}	0.00±0.00^{e}	0.00±0.00^{d}	1.33±1.15^{f}	0.06±0.02^{f}	3.44±0.11^{f}
	Positive control	30.00±2.00^{c}	17.33±3.06^{c}	17.33±2.31^{ab}	21.33±1.15^{b}	14.67±3.06^{b}	53.33±3.06^{c}	1.73±0.06^{c}	134.27±2.23^{c}
	2/3rd LC_{50}	19.33±2.31^{d}	12.67±1.15^{d}	14.00±2.00^{b}	34.00±2.00^{a}	20.00±2.00^{a}	68.00±2.00^{a}	2.23±0.03^{a}	206.67±1.46^{a}
	1/3rd LC_{50}	22.00±3.46^{d}	17.33±1.15^{c}	22.67±4.26^{a}	14.00±2.00^{c}	24.00±2.00^{a}	60.67±4.62^{b}	2.01±0.08^{b}	168.79±0.94^{b}
	1/4th LC_{50}	33.33±4.16^{c}	37.33±1.15^{a}	18.00±4.00^{ab}	7.33±1.15^{d}	4.00±2.00^{cd}	29.33±3.06^{d}	1.11±0.05^{d}	93.60±1.27^{d}

continued.....

	1/5th LC_{50}	40.67±4.16^{b}	32.67±1.15^{b}	13.33±3.06^{b}	7.33±3.06^{d}	6.00±2.00^{c}	26.67±5.03^{e}	1.05±0.11^{e}	89.96±0.50^{e}
70 days	Negative control	96.67±1.15^{a}	2.67±1.15^{d}	0.67±1.15^{e}	0.00±0.00^{e}	0.00±0.00^{e}	0.67±1.15^{d}	0.04±0.02^{d}	3.48±0.09^{e}
	Positive control	23.33±4.16^{d}	26.00±2.00^{c}	12.00±2.00^{bc}	13.33±3.06^{bc}	25.33±3.06^{a}	50.67±2.31^{b}	1.91±0.12^{a}	139.11±1.42^{c}
	2/3rd LC_{50}	20.67±3.06^{e}	22.00±2.00^{c}	23.33±3.06^{a}	15.33±3.12^{b}	18.67±1.15^{b}	57.33±2.31^{a}	1.89±0.05^{a}	168.79±0.94^{a}
	1/3rd LC_{50}	24.67±4.16^{d}	24.67±3.06^{c}	13.33±4.16^{b}	25.33±3.09^{a}	12.00±2.00^{c}	50.67±1.15^{b}	1.75±0.10^{b}	145.77±0.06^{b}
	1/4th LC_{50}	40.67±1.15^{b}	36.00±3.46^{b}	7.33±3.06^{cd}	10.00±2.00^{cd}	5.33±2.31^{d}	22.67±3.06^{c}	1.03±0.08^{c}	73.00±1.39^{d}
	1/5th LC_{50}	30.67±6.11^{c}	51.33±3.06^{a}	6.00±2.00^{d}	8.67±1.15^{d}	3.33±2.31^{de}	18.00±5.29^{c}	1.03±0.17^{c}	72.42±0.27^{d}
84 days	Negative control	97.33±1.15^{a}	2.67±1.15^{e}	0.00±0.00^{e}	0.00±0.00^{c}	0.00±0.00^{e}	0.00±0.00^{f}	0.03±0.01^{f}	3.45±0.33^{e}
	Positive control	27.33±3.06^{d}	16.67±1.15^{d}	21.33±2.31^{a}	12.67±2.31^{b}	22.00±2.00^{a}	56.00±2.00^{a}	1.85±0.09^{a}	136.74±1.34^{a}
	2/3rd LC_{50}	30.00±2.00^{d}	22.00±2.00^{c}	10.67±3.06^{b}	24.00±2.00^{a}	13.33±1.15^{b}	48.00±2.00^{b}	1.69±0.02^{b}	137.46±0.27^{a}
	1/3rd LC_{50}	27.33±2.31^{d}	33.33±1.15^{b}	8.67±1.15^{bc}	20.67±1.15^{a}	10.00±2.00^{c}	39.33±3.06^{c}	1.53±0.10^{c}	113.01±0.70^{b}
	1/4th LC_{50}	36.67±4.16^{c}	42.00±2.00^{a}	5.33±2.31^{cd}	10.67±3.06^{b}	5.33±1.15^{d}	21.33±4.16^{d}	1.06±0.09^{d}	72.26±0.89^{c}
	1/5th LC_{50}	48.67±3.06^{b}	41.33±3.06^{a}	4.00±2.00^{d}	3.33±1.15^{c}	2.67±1.15^{de}	10.00±2.00^{e}	0.70±0.04^{e}	41.62±0.99^{d}

Supplementary file

Exposure time	Treatments	Un-damaged nuclei (%)	Proportions of damaged nuclei (%)				Damaged nuclei (%)	GDI	CTL (µm)
			Type-0	Type-I	Type-II	Type-III		Type-IV	
14 days	Negative control	94.67±2.31^{a}	3.33±1.15^{d}	2.00±2.00^{d}	0.00±0.00^{d}	0.00±0.00^{d}	2.00±2.00^{f}	0.07±0.04^{f}	3.49±0.09^{f}
	Positive control	33.33±3.06^{b}	12.00±3.46^{c}	11.33±1.15^{c}	21.33±1.15^{a}	22.00±2.00^{a}	54.67±1.15^{b}	1.87±0.06^{b}	131.92±1.96^{c}
	2/3rd LC_{50}	10.00±3.46^{d}	28.00±5.29^{b}	27.33±3.06^{a}	10.00±2.00^{b}	24.67±4.16^{a}	62.00±2.00^{a}	2.11±0.05^{a}	180.46±0.79^{a}
	1/3rd LC_{50}	31.33±3.06^{b}	17.33±2.31^{c}	20.00±2.00^{b}	17.33±4.62^{a}	14.00±4.00^{b}	51.33±1.15^{c}	1.65±0.08^{c}	137.94±0.72^{b}
	1/4th LC_{50}	34.67±1.15^{b}	32.00±2.00^{b}	14.00±2.00^{c}	10.67±3.06^{b}	8.67±3.06^{c}	33.33±2.31^{d}	1.27±0.02^{d}	103.552±0.36^{d}
	1/5th LC_{50}	24.67±4.16^{c}	60.67±5.03^{a}	5.33±1.15^{d}	5.33±1.15^{c}	4.00±2.00^{cd}	14.67±1.15^{e}	1.03±0.05^{e}	46.83±0.73^{e}
28 days	Negative control	94.67±1.15^{a}	4.67±1.15^{e}	0.67±1.15^{d}	0.00±0.00^{e}	0.00±0.00^{e}	0.67±1.15^{e}	0.06±0.02^{f}	3.43±0.05^{f}
	Positive control	30.00±2.00^{c}	14.67±1.15^{d}	21.33±2.31^{a}	12.67±1.15^{c}	21.33±1.15^{b}	55.33±2.31^{b}	1.18±0.05^{d}	135.75±5.05^{c}
	2/3rd LC_{50}	11.33±1.15^{b}	12.67±3.06^{d}	24.67±1.15^{a}	18.67±3.06^{b}	32.67±1.15^{a}	76.00±3.46^{a}	2.49±0.08^{a}	224.82±0.16^{a}
	1/3rd LC_{50}	17.33±2.31^{d}	22.67±3.06^{c}	11.33±2.31^{c}	28.67±2.31^{a}	20.00±2.00^{b}	60.00±2.00^{b}	2.11±0.08^{b}	197.36±0.95^{b}
	1/4th LC_{50}	27.33±4.16^{c}	30.67±4.62^{b}	16.67±3.06^{b}	13.33±3.06^{c}	12.00±2.00^{c}	42.00±3.46^{c}	1.52±0.10^{c}	128.63±0.41^{d}
	1/5th LC_{50}	36.00±2.00^{e}	44.00±2.00^{a}	8.67±1.15^{c}	8.00±2.00^{d}	3.33±2.31^{d}	20.00±3.45^{d}	0.99±0.12^{e}	73.53±0.34^{e}
42 days	Negative control	95.33±1.115^{a}	3.33±1.15^{e}	1.33±1.15^{d}	0.00±0.00^{e}	0.00±0.00^{e}	1.33±1.15^{e}	0.06±0.02^{e}	3.38±0.01^{f}
	Positive control	25.33±2.31^{cd}	26.67±3.06^{a}	10.67±3.06^{c}	14.00±2.00^{c}	23.33±2.31^{b}	48.00±2.00^{c}	1.83±0.01^{c}	131.27±1.29^{d}
	2/3rd LC_{50}	8.00±2.00^{e}	8.00±2.00^{d}	23.33±4.16^{a}	27.33±4.16^{a}	33.33±3.06^{a}	84.00±3.46^{a}	2.70±0.15^{a}	238.52±1.05^{a}
	1/3rd LC_{50}	21.33±1.15^{d}	12.67±3.06^{c}	14.00±3.46^{cd}	19.33±3.06^{b}	32.67±2.31^{a}	66.00±3.46^{b}	2.29±0.06^{b}	204.67±1.46^{b}
	1/4th LC_{50}	27.33±3.06^{c}	18.67±3.06^{b}	18.67±1.15^{ab}	20.67±4.16^{b}	14.67±3.06^{c}	54.00±6.00^{c}	1.77±0.16^{c}	163.84±0.50^{c}
	1/5th LC_{50}	46.00±2.00^{b}	30.00±2.00^{a}	11.33±3.06^{c}	8.00±2.00^{d}	4.67±1.15^{d}	24.00±4.00^{d}	0.95±0.09^{d}	71.93±1.17^{e}

showed various changes in the normal behaviour, cytology, physiology and histological parameters of fish (Bakshi and Panigrahi, 2018; Arunkumar *et al*., 2006). Chromium is a transition metal that enters the cell *via* sulphate ion transporters and is reduced to trivalent chromium through reactive intermediate forms to cause oxidative stress (Wise *et al*., 2006). Chromium binding formed DNA-DNA or DNA-protein cross-linking, strand breaks and specifically Cr-DNA adducts that count for most of the chromium induced mutagenic effects (Zhitkovich, 2005). During the present research work, all three fish species showed significantly variable DNA damage after exposure to various concentrations of chromium, however, *C. mrigala* exhibited higher DNA damage followed by *L. rohita* and *C. catla*. Similar results were reported by Kousar and Javed (2015) that exposure to sub-lethal concentrations (17, 25, 33 and 55% of LC_{50}) of zinc, copper and arsenic induced significant genotoxic effects in peripheral erythrocytes of four cyprinids that followed the order: *C. mirgala* >*L. rohita* >*C. idella* >*C. catla*. The genetic damage index (GDI) values for three species of fish exposed to metals varied significantly also. All three major carps showed variable behaviour towards chromium toxicity due to their different physiological needs and species specificity to interact against heavy metals. Moreover, DNA damage caused by heavy metals suggested a serious concern towards their potential danger to the survival of carps in the natural aquatic habitats (Latif and Javed, 2019).

During the present investigation, all three major carps showed a concomitant increase in DNA damage in their peripheral erythrocytes with an increase in metallic ion concentration of chromium as maximum DNA damage in terms of GDI and CTL was observed in $2/3^{rd}$ of LC_{50} for all the fish species under study. The transition metals *viz*. copper and chromium induce oxidative DNA damage, which depends upon the interaction of metals and DNA, along with the valency of transition ions (Moriwaki *et al*., 2008). The toxicity of transition metals is due to their higher potential to act as catalysts in the production of ROS through Haber-Weiss/Fenton reactions, resulting in potentially damaging DNA modifications (Aboul-Ela *et al*., 2011). These ROS cause oxidation of de-oxyribose or indirectly affect excision repair mechanisms that leads to single-strand breakage and ends up in DNA double-strand breakage during replication (De Zio *et al*., 2012). The higher %age of tail DNA in the RBCs of *L. rohita* after exposure to sub-lethal concentrations *i.e*. 29.5, 59.0 and 88.5 mg/L of chromium reported by Nagpure *et al*. (2014). The chromium exposure dose and time dependent DNA damage in the RBCs of *C. catla* observed by Arunachalam *et al*. (2013). Exposure to sub-lethal concentration (50% of LC_{50}) of chromium for 21 days caused significantly higher tail moments as compared to the 7^{th} and 14^{th} days of exposure. They described that the possible mechanism behind this damage was Cr-DNA adduct formation and DNA replication inhibition by hexavalent chromium (Nickens *et al*., 2010). Other symptoms like intra-strand cross-links and strand breaks in salmon sperm DNA with extensive DNA strand breakage have been evident in salmon fish when exposed to 1 mM chromium concentration.

Conclusion

Hexavalent chromium induced significant dose and time dependent DNA damage in the erythrocytes of three major carps. Among the three fish species, *C. mrigala* exhibited significantly higher DNA damage in terms of percentage damaged nuclei, genetic damage index and comet tail length both in dose and time dependent exposures followed by *L. rohita* and *C. catla*. Among various exposure durations, DNA damage was observed higher at 56 days exposure which then starts decreasing as the DNA repair mechanism in fish starts working towards repair whereas exposure dose-dependent DNA showed higher damage after exposure to $2/3^{rd}$ of LC_{50} of chromium to all three fish species followed by positive control.

Conflict of Interest. The authors declare that they have no conflict of interest.

References

Aboul-Ela, H.M., Saad, A.A., El-Sikaily, A.M.A., Zaghloul, T.I. 2011. Oxidative stress and DNA damage in relation to transition metals overload in Abu-Qir Bay. *Egyptian Journal of Genetic Engineering and Biotechnology*, **9:** 51-58.

Ali, D., Nagpure, N.S., Kumar, S., Kumar, R., Kushwaha, B. 2008. Genotoxicity assessment of acute exposure of chlorpyrifos to freshwater fish *Channa punctatus* (Bloch) using micro-nucleus assay and alkaline single-cell gel electrophoresis. *Chemosphere*, **71:** 1823-1831.

Arunachalam, K.D., Annamalai, S.K., Kuruva, J.K. 2013. *In-vivo* evaluation of hexavalent chromium

induced dna damage by alkaline comet assay and oxidative stress in *Catla catla*. *American Journal of Environmental Sciences*, **9:** 470-482.

Arunkumar, R.I., Rajashekaran, P., Michael, R.D. 2006. Differential effect of chromium compounds on the immune response of the African mouth breeder Oreochromis mossambicus (Peter). *Fish Shellfish Immunology*, **10:** 667-676.

Bakshi, A., Panigrahi, A.K. 2018. A comprehensive review on chromium induced alterations in fresh-water fishes. *Toxicology Reports*, **5:** 440-447.

Bolognesi, C., Cirillo, S. 2014. Genotoxicity biomarkers in aquatic bioindicators. *Current Zoology*, **60:** 273-284.

De Zio, D., Bordi, M., Cecconi, F. 2012. Oxidative DNA damage in neurons: implication of Ku in neuronal homeostasis and survival. *International Journal of Cell Biology*, **2012:** Article ID 752420.

Emere, M.C., Dibal, D.M. 2013. Metal accumulation in some tissues/organs of a fresh water fish (*Clarias gariepinus*) from some polluted zones of river Kaduna. *Journal of Biological Chemistry*, **3:** 112-118.

Frenzilli, G., Lyons, B.P. 2013. The comet assay in marine animals. *Methods in Molecular Biology*, **1044:** 363-372.

Javed, M. 2013. Chronic effects of nickel and cobalt on fish growth. *International Journal of Agriculture and Biology*, **15:** 575-579.

Kehinde, O.H., Ajibola, I.O., Isaac, S., Segun, O.O. 2016. Determination of heavy metal genotoxicity and their accumulation pattern in different fish organs of selected fish species collected from the Asa river, Ilorin, Kwara state, Nigeria. *The Journal of Applied Sciences and Environmental Management*, **20:** 735-745.

Kousar, S., Javed, M. 2015. Diagnosis of metals induced DNA damage in fish using comet assay. *Pakistan Veterinary Journal*, **32:** 168-172.

Latif, F., Javed, M. 2019. Inter-specific differences in the sensitivity, accumulation and antioxidant capacities of three cyprinids exposed to heavy metals mixture. *Pakistan Journal of Zoology*, **51:** 809-816.

Moriwaki, H., Osborne, M.R., Phillips, D.H. 2008. Effects of mixing metal ions on oxidative DNA damage mediated by a Fenton-type reduction. *Toxicology In Vitro*, **22:** 36-44.

Nassour, J., Martien, S., Martin, N., Deruy, E., Tomellini, E., Malaquin, N., Bouali, F., Sabatier, L., Wernert, N., Pinte, S., Gilson, E., Pourtier, A., Pluquet, O., Abbadie, C. 2016. Defective DNA single-strand break repair is responsible for senescence and neoplastic escape of epithelial cells. *Natural Communications*, **7:** 10399.

Nagpure, N.S., Srivastava, R., Kumar, R., Kushwaha, B., Srivastava, S.K., Kumar, P., Dabas, A. 2014. Assessment of genotoxic and mutagenic potential of hexavalent chromium in the freshwater fish *Labeo rohita* (Hamilton, 1822). *Drug and Chemical Toxicology*, **38:** 9-15.

Nickens, K.P., Patierno, S.R., Ceryak, S. 2010. Chromium genotoxicity: a double-edged sword. *Chemico-Biological Interactions*, **188:** 276-288.

Praveena, M., Sandeep, V., Kavitha, N., Jayantha Rao, K. 2013. Impact of tannery effluent, chromium on hematological parameters in a fresh water fish, *Labeo rohita* (Hamilton), *Research Journal of Animal, Veterinary and Fishery Sciences*. 1.

Singh, V., Singh, N., Verma, M., Kamal, R., Tiwari, R., Chivate, M.S., Rai, S.N., Kumar, A., Singh, A., Singh, M.P., Yamanu, E., Mishra, Y. 2022. Hexavalent-chromium-induced oxidative stress and the protective role of antioxidants against cellular toxicity. *Antioxidants*, **11:** 2375.

Singh, V., Mishra, V. 2021. Microbial removal of Cr (VI) by a new bacterial strain isolated from the site contaminated with coal mine effluents. *Journal of Environmental Chemical Engineering*, **9:** 106279.

Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L. 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research*, **175:** 184-191.

Sultana, T., Butt, K., Sultana, S., Al-Ghanim, K.A., Mubashra, R., Bashir, N., Ahmed, A., Ashraf, A., Mahboob, S. 2016. Histopathological changes in liver, gills and intestine of *Labeo rohita* inhabiting industrial waste contaminated water of river Ravi. *Pakistan Journal of Zoology*, **48:** 1171-1177.

Ullah, A., Rehman, H.U., Saeed, W., Quraish, M.F., Ullah, Z., Haroon Ur Rehman, R., Ahmad, W., Saeed, K. 2017. Determination of 96-hr Lc50 value of cadmium for a fish, *Labeo rohita*. *Journal of Entomology and Zoology Studies*, **4:** 380-382.

Valavanidis, A., Vlahogianni, T., Dassenakis, M., Scoullos, M. 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicology and Environmental Safety*, **64:** 178-189.

Varotto, L., Domeneghetti, S., Rosani, U., Manfrin, C., Cajaraville, M.P., Raccanelli, S., Pallavicini, A., Venier, P. 2013. DNA damage and transcriptional changes in the gills of *Mytilus galloprovincialis* exposed to nanomolar doses of combined metal salts (Cd, Cu, Hg). *PLoS One*, **8:** e54602.

Veeraiah, K., Venkatrao, G., Vivek, C., Hymaranjani, G. 2013. Heavy metal, cadmium chloride induced biochemical changes in the Indian major carp *Cirrhinus mrigala* (Hamilton). *International Journal of Biomassays*, **2:** 1028-1033.

Wang, J., Liu, C., Zhao, Y., Wang, J., Li, J., Zheng, M. 2023. Selenium regulates Nrf_2 signaling to prevent hepatotoxicity induced by hexavalent chromium in broilers. *Poultry Science*, **102:** 102335.

Wise, S.S., Holmes, A.L., Wise, J.P. 2006. Particulate and soluble hexavalent chromium are cytotoxic and genotoxic to human lung epithelial cells. Mutation Research. *Genetic Toxicology and Environmental Mutagenesis*, **610:** 2-7.

Zhang, Y., Long, C., Hu, G., Hong, S., Su, Z., Zhang, Q., Jia, G. 2023. Two-week repair alleviates hexavalent chromium-induced hepatotoxicity, hepatic metabolic and gut microbial changes: a dynamic inhalation exposure model in male mice. *Science Total Environment*, **857:** 159429.

Zhitkovich, A. 2005. Importance of chromium-DNA adducts in mutagenicity and toxicity of chromium(VI). *Chemical Research in Toxicology*, **18:** 3-11.