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Research Paper

Relationship between genotoxicity and oxidative stress induced by mercury on common carp (*Cyprinus carpio*) tissues

Sandra García-Medina[a,*], Marcela Galar-Martínez[a,*], Leobardo Manuel Gómez-Oliván[b], Karina Ruiz-Lara[a], Hariz Islas-Flores[b], Eloy Gasca-Pérez[a]

[a] Laboratorio de Toxicología Acuática, Sección de Graduados e Investigación, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Plan de Ayala y Carpio s/n, 11340 México D.F., México

[b] Laboratorio de Toxicología Ambiental, Facultad de Química, Universidad Autónoma del Estado de México, Paseo Colón intersección Paseo Tollocan s/n, Col. Residencial Colón, 50120 Toluca, Estado de México, México

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ABSTRACT

Mercury is one of the most toxic metals in aquatic systems since it is able to induce neurobehavioral disorders as well as renal and gastrointestinal tract damage. The common carp *Cyprinus carpio* is an important species from both an ecological and economic viewpoint as it is consumed in many countries, the top producers being Mexico, China, India and Japan. The present study aimed to evaluate the relation between Hg-induced oxidative stress and genotoxicity in diverse tissues of *C. carpio*. Specimens were exposed to 0.01 mg Hg/L (the maximum permissible limit for aquatic life protection), and lipid peroxidation, protein carbonyl content and the activity of antioxidant enzymes were evaluated at 96 h. Micronuclei frequency and DNA damage by comet assay were determined at 12, 24, 48, 72 and 96 h. Hg induced oxidative stress and genotoxicity on exposed fish, since inhibition of antioxidant enzymes activity and increases in lipid peroxidation, DNA damage and micronuclei frequency occurred. Blood, gill and liver were more susceptible to oxidative stress, while blood were more sensitive to genotoxicity. In conclusion, Hg at concentrations equal to the maximum permissible limit for aquatic life protection induced oxidative stress and genotoxicity on *C. carpio*, and these two effects prove to be correlated.

1. Introduction

Metals such as lead, mercury (Hg) and cadmium as well as the metalloid arsenic are a potentially significant health hazard for living organisms, particularly in aquatic ecosystems (Has-Schön et al., 2015). The presence of these metals in water bodies has increased significantly in recent decades since they are extensively used in agriculture as well as chemical and industrial processes (Chandran et al., 2005). For instance, environmental contamination by Hg is due primarily to anthropogenic activities and sources such as dumping of urban waste, agricultural products, mining operations, fossil fuel combustion and industrial discharges (Kalafatić et al., 2004; Horowitz et al., 2014). Organic and inorganic species of mercury are widely distributed in aquatic ecosystems, and have been detected in the sediment and water of diverse aquatic systems around the world (Table 1) almost always above the maximum limits established.

Once it enters an ecosystem, Hg can be uptaken by living organisms, inducing toxic effects. Most importantly, it is usually bioaccumulated and biomagnified, and is therefore considered one of the most hazardous metals for these ecosystems (Lavoie et al., 2013). In fish as well as mammals, it can induce neurobehavioral disorders as well as renal and gastrointestinal tract damage (Kakkar and Jaffery, 2005). In fish such as *Salmo salar*, *Pomatoschistus microps*, *Cyprinus carpio*, *Liza aurata* and *Ictalurus melas* it has been found to bioaccumulate in the gills, kidneys, liver, muscle and brain, altering antioxidant enzymes activity, increasing protein carbonyl content (PCC), lipid peroxidation (LPX) and the levels of biomolecules rich in sulfhydryl groups – reduced glutathione (GSH) and metallothioneins – and inducing natatory, reproductive, feeding and sensory behavior disorders (Berntssen et al., 2003; Elia et al., 2003; Guilherme et al., 2008; Navarro et al., 2009; Vieira et al., 2009; Gómez-Oliván et al., 2017).

Cellular damage induced by metals such as Hg has been associated with production of reactive oxygen species (ROS). Fish, like all other aerobic organisms, are susceptible to attack by ROS and have consequently developed antioxidant defenses, particularly in the form of adapted enzymes such as superoxide dismutase (SOD), catalase (CAT),

* Corresponding authors.
E-mail addresses: sandygamed@gmail.com (S. García-Medina), marcela_galar_martinez@hotmail.com, mgalarm@ipn.mx (M. Galar-Martínez).

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Table 1
Concentration of mercury in sediments and water from various bodies around the world and permissible limits values.

Location	Hg concentration (ng/kg in sediments or ng/L in water)	Permissible limits
Sediments from Punnakayal estuary (India), (Magesh et al., 2013)	3300	0.01 mg/kg (BIS, 2012)
Sediments from Minamata bay (Japan), (Matsuyama et al., 2016)	29600	1 mg/kg (JPHA, 2001)
Water from Minamata bay (Japan), (Guentzel et al., 2007)	1.3–4.3 (2002)	0.0005 mg/L (JPHA, 2001)
Water from Minamata bay (Japan), (Guentzel et al., 2007)	0.84–1.64 (2005)	0.0005 mg/L (JPHA, 2001)
Water from Papaloapan basin (Mexico), (Tomiyasu et al., 2008)	1.0–12.7	0.01 mg/L (NOM-001-ECOL-1996)
Water from Yellow river (China), (Hou et al., 2016)	0.03–0.06	0.001 (irrigation) and 0.0005 (fish culture) mg Hg/L (Zhang and Wong, 2007)
Water from Yamuna river (India), (Rahman and Singh 2016)	80–100	0.001 mg/L (UNEP, 2009)

glutathione peroxidase (GPx), glutathione reductase and peroxiredoxins. An increasing number of studies on the antioxidant defense systems of fish have established the usefulness of these enzymes as biomarkers of effect in environmental system monitoring (Van der Oost et al., 2003; Santovito et al., 2012; Tolomeo et al., 2016; Lushchak, 2016). Antioxidant enzyme activity can be either induced or inhibited in the presence of contaminants. Such response depends on the intensity and duration of exposure as well as the susceptibility of the species exposed. The enzyme SOD is the first enzyme involved in the process of ROS detoxification; it catalyzes dismutation of the superoxide radical ($O_2^{-}\cdot$) to hydrogen peroxide (H_2O_2). The latter is broken down into water and molecular oxygen by CAT or GPx. In turn, GPx catalyzes the reduction of lipid hydroperoxides (LOOHs) to alcohols (LOHs); two GSH molecules are oxidized in this reaction, being subsequently reduced again by glutathione reductase (Monteiro et al., 2010).

In recent years, Hg has been recognized as a cytogenotoxic agent. This property has been related to its pro-oxidant capacity and the fact that it can modify proteins that make up the cytoskeleton or are involved in DNA repair. Most of these studies have been carried out on mammals and have tested a large variety of tissues and concentrations as well as inorganic and organic species of this metal (Schmid et al., 2007; Crespo-López et al., 2009). However, some studies have been carried out on fish, such as those by Nepomuceno et al. (1997), who detected an increase in micronuclei (MNi) frequency in peripheral blood erythrocytes of *C. carpio* exposed to metallic mercury, and Gómez-Oliván et al. (2017), who found induction of DNA damage and cytotoxicity when this metal is present as a mixture with aluminum and iron. The genotoxicity of environmental contaminants can be monitored using a broad range of *in vitro* and *in vivo* biomarkers of DNA damage. The micronucleus test and comet assay have turned out to be excellent tools for use in monitoring environmental genotoxicity in fish (Selvi et al., 2013).

MNi are whole chromosomes or chromosome fragments that were not incorporated into the nucleus of daughter cells during cell division and are apparent as small, round dark structures. The micronucleus test has shown a high potential for detection of clastogens, which induce acentric fragments, as well as aneugens, which induce chromosome loss mainly by interference with the mitotic spindle in aqueous media, in diverse species (Kan et al., 2012; Obiakor et al., 2014). MNi are formed during mitosis regardless of the type of damage that may have occurred during the cell cycle, and therefore DNA lesions are expressed in these structures only after one cell cycle and depend on the percentage of dividing cells and duration of the process (Fenech, 1997). On the other hand, the comet assay has been applied in species already used in biomonitoring and has proved to be a sensitive system for screening the potential genotoxicity of chemicals and complex mixtures (Bhowmik and Patra, 2012). This assay detects single-strand DNA breaks, alkali-labile sites and crosslinking (Tice et al., 2000).

The common carp *C. carpio* is one of the most abundant fish species in freshwater systems. It is commercially cultured and is frequently consumed by humans. According to the Food and Agriculture Organization of the United Nations, in 2002 common carp accounted for 14% of the total global freshwater aquaculture production. The overall growth rate in production was 9.5% annually between 1985 and 2002, while in the last decade it increased 10.4% annually. Furthermore, since this species is able to bioaccumulate metals, it is also an adequate bioindicator of environmental contamination by these agents (Brumbaugh et al., 2005; García-Medina et al., 2010; FAO, 2004).

The present study aimed to evaluate oxidative stress induced in gill, brain, liver and blood of *C. carpio* exposed to Hg at a concentration equal to the maximum permissible limit for aquatic life protection, and the relationship between genotoxicity elicited at chromosome level and DNA strand breakage in tissues evidencing higher levels of oxidative damage.

2. Materials and methods

2.1. Chemicals

Mercury chloride ($HgCl_2$) and reagents used in LPX, PCC and enzyme activity determination as well as comet assay and the micronucleus test were purchased from Sigma Chemical (St. Louis, MO). Nitric acid (HNO_3), Ultrex ultrapure reagent, was obtained from J.T. Baker (Phillipsburg, NJ).

2.2. Specimen procurement and acclimation

Common carp (*Cyprinus carpio*) specimens weighing 46.1 ± 0.9 g and 10.2 ± 0.4 cm long were obtained from the aquaculture center in Tiacaque (State of Mexico, Mexico), transported to the laboratory and acclimated for two months. Fish were maintained in 120 L glass tanks (20 fish per tank) equipped with a constant filtration and circulation system, at 20 ± 2 °C, with 80–90% oxygen concentration, pH 7.5–8.0, and a 12 h:12 h light:dark photoperiod, and were fed Pedregal Silver Cup^{MR} every third day.

2.3. Quantification of Hg

Five carp, each weighing 35–40 g, were placed in exposure systems such as those described below for oxidative stress determination and exposed to 0.01 mg Hg/L for 96 h. The assay was performed in triplicate. At the end of the exposure period, fish were euthanized, frozen in liquid nitrogen and macerated to homogenize tissues. A 0.5 g sample of each fish was digested in a CEM Mars 5 microwave oven equipped with an HP-500 closed-vessel digestion system. Each digestion vessel was added 10 mL concentrated HNO_3, then allowed to rest for 15 min. Digestion conditions were: 300 W (100% power), ramp time 15 min,

400 psi, 210 °C, hold time 10 min. Each 1 mL water sample was supplemented with 10 mL concentrated HNO_3 prior to digestion by the same procedure. Hg was quantified in a SpectrAA 50 atomic absorption spectrophotometer coupled to a hydride generator. Hg concentration was determined by interpolation on a standard curve with dilutions of 0.005, 0.01, 0.015 and 0.02 ppm Hg at 253.7 nm wavelength. The bioconcentration factor (BCF) was calculated as the ratio Hg concentration in the specimen to Hg concentration in water from the corresponding exposure system.

2.4. Evaluation of oxidative stress

The test concentration used is equal to the maximum permissible limit in Mexico for aquatic life protection: 0.01 mg Hg/L (NOM-001-ECOL-1996). Two fish were exposed in triplicate (N = 6) in a static model without renewal, in plastic fish tanks with 10 L tap water. A control group was set up with tap water alone. Test system water had the following physicochemical characteristics: dissolved oxygen 6.470 ± 5 mg/L, ammonia concentration 0.3270 ± 2 mg/L, nitrate 0.2670 ± 001 mg/L, temperature 20 ± 2 °C, 90–100% oxygen saturation and pH 7.5–8.0. After 96 h of exposure, blood samples were obtained from the caudal vessel; specimens were euthanized and the liver, brain and gills were removed. Tissue samples were homogenized in 1 mL phosphate buffered saline (PBS) pH 7.4. Blood samples were diluted 1:5 with PBS and homogenized for 30 s. All samples were centrifuged at 16,000 × *g* for 15 min at 4 °C. The supernatant was used to determine all oxidative stress biomarkers except LPX.

2.4.1. Determination of superoxide dismutase activity

SOD activity was determined by the Misra and Fridovich (1972) method. To 20 µL of supernatant was added 150 µL carbonate buffer solution (50 mM sodium carbonate, 0.1 mM EDTA) pH 10.2, plus 100 µL adrenaline (30 mM), and absorbance was read at 480 nm at 30 s and 5 min. Activity was determined by interpolation on a SOD type curve (bovine erythrocyte Cu/Zn SOD). Results were normalized against total protein content.

2.4.2. Determination of catalase activity

CAT activity was determined by the Radi et al. (1991) method. To 20 µL of supernatant was added 1 mL isolation buffer solution (0.3 M sucrose, 1 mM EDTA, 5 mM HEPES, 5 mM KH_2PO_4) and 0.2 mL of a 20 mM H_2O_2 solution. Absorbance was determined at 240 nm, at 0 and 60 s. Activity per minute was calculated using the molar extinction coefficient (MEC) of H_2O_2 (0.043/mM/cm). Results were normalized against total protein content.

2.4.3. Determination of glutathione peroxidase activity

GPx activity was determined using the method of Paglia and Valentine (1967): to 100 µL of supernatant was added 900 µL reaction buffer solution (50 mM K_2HPO_4, 50 mM KH_2PO_4, 3.5 mM reduced glutathione, 1 mM sodium azide, 2 U glutathione reductase, 0.12 mM NADPH pH 7.0) and 200 µL H_2O_2 (0.8 mM). Absorbance was read at 340 nm, at 0 and 60 s. Activity was calculated using the MEC of NADPH (6.2/mM/cm). Results were normalized against total protein content.

2.4.4. Determination of lipid peroxidation

LPX was determined using the Büege and Aust (1979) method. To 300 µL of homogenate was added 1 mL Tris-HCL buffer solution (150 mM) pH 7.4, incubating at 37 °C for 30 min. Next, 2 mL TBA-TCA reagent (0.375% thiobarbituric acid in 15% trichloroacetic acid) was added prior to incubating at 37 °C for 45 min, followed by centrifugation at 885 x g and −4 °C for 15 min. Absorbance was read at 535 nm and results were expressed as nM malondialdehyde (MDA)/mg protein using the MEC of 1.56 × 10^5/M/cm.

2.4.5. Determination of protein carbonyl content

PCC was determined using the Levine et al. (1994) procedure, modified as follows: to 100 µL of supernatant was added 150 µL of 10 mM dinitrophenylhydrazine in HCl (2 M) prior to incubating at room temperature for 1 h in the dark. Next, 500 µL of 20% TCA was added; the sample was allowed to rest for 15 min at 4 °C, then centrifuged at 12,000 × *g* for 5 min. The bud was washed three times with 1:1 ethanol:ethyl acetate, dissolved in 1 mL guanidine (6 M) in a potassium phosphate buffer solution (2 mM; pH adjusted to 2.3 with trifluoroacetic acid), and incubated at 37 °C for 30 min. Absorbance was read at 366 nm, and results were expressed as nM reactive carbonyls (C═O) formed/mg protein using the MEC of 21,000/M/cm.

2.4.6. Determination of total protein content

To 25 µL of supernatant was added 75 µL deionized water and 2.5 mL Bradford reagent (0.05 g Coomassie Blue dye, 25 mL of 96% ethanol and 50 mL H_3PO_4, in 500 mL deionized water). The test tubes were shaken and allowed to rest for 5 min prior to reading of absorbance at 595 nm. Total protein content was determined by interpolation on a bovine serum albumin standard curve and was used to normalize the data (Bradford, 1976).

2.5. Evaluation of genotoxicity

To determine the genotoxic potential of Hg, six carp were exposed to 0.01 mg Hg/L for 12, 24, 48, 72 and 96 h. An Hg-free control system was set up and the test was performed in triplicate. At the end of each exposure time, blood samples were obtained and diluted 1:4 with PBS. Fish were euthanized and dissected; the liver and gills were removed, pestle-pressed through a very fine mesh into a mortar with 0.5 mL isotonic solution (0.9% NaCl) and gently homogenized for use in the micronucleus test. Tissues used in the comet assay were macerated in a mortar with 500 µL PBS at 4 °C and gently homogenized for 1 min. Cellular viability (92.5 ± 3.20%) was confirmed using 0.4% trypan blue. This bioassay was performed on tissues with at least one correlation value between biomarkers of oxidative damage and antioxidant defenses greater than 0.9.

2.5.1. Micronucleus test

Smears of the cell suspension (whole blood or macerated organ) were fixed in pure ethanol for 3 min, stained with a 10% solution of Giemsa in PBS (pH 6.7) for 10 min and rinsed in tap water. A total of 1000 cells were quantified per treatment in an optical microscope (Motic BA210, immersion lens) and MNi frequency was expressed as the number of nucleated cells per 1000 cells. To determine presence of MNi, the following criteria were used: small nuclei not attached to the main nucleus, stain color and intensity similar to those of the main nucleus, and diameter 1/5–1/20 of main nucleus diameter (Bolognesi and Cirillo, 2014).

2.5.2. Comet assay

DNA damage was evaluated by comet assay as proposed by Tice et al. (2000), with modifications. The slide containing the cells was prepared 1 h prior to obtaining the sample. A 100 µL layer of 1% normal agarose was placed on the slide. Next, 10 µL of cell suspension was mixed with 75 µL of 0.7% normal agarose, and 50 µL of this mixture were placed on the initial agarose layer. To extract DNA, slides were placed in a Coplin jar with lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1% Triton X-100, at pH 10) for 1 h at 4 °C. Slides were placed in the electrophoresis chamber for 20 min with an alkaline solution (300 mM NaOH and 1 mM EDTA) at pH ≥ 13. Electrophoresis was performed at 300 mA, 25 V, and pH > 13 for 20 min, and the process was stopped with neutralizing buffer (0.4 M Trizma base) at pH 7.4. The DNA was stained with 50 µL ethidium bromide and examined in an epifluorescence microscope (Motic BA410) equipped with digital camera (Moticam Pro CCD). A total of

Table 2
Pearson's correlation analysis of oxidative damage with antioxidant defenses in tissues of *C. carpio* exposed to Hg during 96 h.

Biomarkers oxidative stress	Liver		Gill		Blood		Brain	
	LPx	PCC	LPx	PCC	LPx	PCC	LPx	PCC
SOD	**0.990**	0.284	**0.530**	**0.921**	**−0.913**	**0.931**	0.413	**0.764**
CAT	−0.199	**−0.597**	**0.511**	**0.696**	0.438	−0.146	**−0.791**	**0.615**
GPx	**−0.820**	**0.907**	−0.064	−0.413	**0.506**	**−0.540**	−0.436	−0.068

Correlation coefficients > 0.5 are statistically significant (shown in bold, p ≤ 0.05).

100 measurements were made per replicate, and damage index (DI) – the ratio comet tail (T) to nuclear diameter (N) – was obtained (Cariño-Cortés et al., 2010).

2.6. Statistical analysis

All values were expressed as the mean ± SEM. Significant differences in oxidative stress between exposed and control groups were determined with a *t*-test. Nonparametric two-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls *post-hoc* multiple comparisons test were used on MNi and damage index results. The level of significance was set at $p < 0.05$. Pearson's correlation analysis was used to find potential correlations between oxidative damage and antioxidant defenses (Table 2), and Hg concentration in specimens, oxidative stress biomarkers and biomarkers of genotoxicity (Table 3). Sigmaplot v12.3 (Systat Software, Richmond, CA) was used.

3. Results

3.1. Quantification of Hg

After 96 h, Hg concentration in exposed specimens was 0.0037 ± 0.0003 mg/kg, while in water from exposure systems it was 0.0052 ± 0.0003 mg/L; the BCF was 0.7168 ± 0.0861.

3.2. Evaluation of oxidative stress

Fig. 1A shows SOD activity results. A nonsignificant increase of 69% in gill and a significant reduction of 78% in blood relative to the control group ($p < 0.05$) were found in exposed fish, while SOD activity values in liver and brain were similar to those in control fish.

CAT activity is shown in Fig. 1B. A tendency towards increased activity (73%) was observed in liver with respect to the control group ($p < 0.05$), while in brain, gill and blood nonsignificant reductions of respectively 76, 63 and 45% were found.

Fig. 1C shows GPx activity results. Significant reductions of 49, 33 and 83% relative to the control group ($p < 0.05$) were found respectively in liver, gill and blood; unlike brain, in which GPx activity levels were closely similar in all study groups.

LPX results are shown in Fig. 1D. A significant increase of 292% was observed in blood ($p < 0.05$). Values in all other tissues did not differ significantly from control group values, but a tendency towards increased LPX was seen in gill and liver (67 and 94% respectively).

Fig. 1E shows PCC results. No significant differences with respect to the control group were observed in liver, gill or blood, but a significant increase of 82% ($p < 0.05$) occurred in brain.

3.3. Evaluation of genotoxicity

Mean MNi frequency in gill cells, hepatocytes and erythrocytes is shown in Fig. 2. Gill cells and hepatocytes recorded similar MNi values at the various exposure times, with a tendency for these values to increase at 48 h. In erythrocytes, a significant increase with respect to the control group occurred from 12 h on, peaking at 48 h ($p < 0.05$).

Comet assay results are summarized in Fig. 3. Significant increases in DNA damage were observed in gill and liver from 12 h on, reaching a maximum value at 48 h in gill and decreasing thereafter ($p < 0.05$). In liver, decreases occurred at 24 and 48 h, followed by an increase at 72 h and a further decrease at 96 h; DNA damage in this organ remained always above control group values ($p < 0.05$). In blood (erythrocytes), DI increased significantly at 12 h, with similar values being maintained thereafter ($p < 0.05$).

3.4. Correlation analysis

Table 2 shows correlation results between oxidative damage (LPX and PCC) and antioxidant defenses (SOD, CAT and GPx) in exposed carps after 96 h. Correlation values > 0.5 were found between oxidative damage and enzymes activity, finding values greater than 0.9 only in liver, gills and blood, so it was decided to perform genotoxicity tests only in those tissues.

Table 3 shows correlation results between oxidative stress and genotoxicity values in common carp tissues and Hg concentration in specimens after 96 h. Correlation values > 0.5 were found between antioxidant enzymes activity and DNA damage determined by comet assay in liver and blood. As regards cytogenetic damage, a > 0.5 value was found between MNi frequency in gill and blood, and Hg concentration in carp.

Table 3
Pearson's correlation analysis of genotoxic damage with biomarkers of oxidative stress and level of Hg at 96 h in tissues of *C. carpio*.

Biomarkers oxidative stress	Liver		Gill		Blood	
	Comet assay	MNi test	Comet assay	MNi test	Comet assay	MNi test
SOD	0.104	**0.873**	0.468	−0.300	**0.507**	−0.233
CAT	**0.939**	0.214	−0.217	0.181	**0.582**	−0.427
GPx	−0.197	**0.755**	−0.395	0.390	0.303	0.048
LPx	−0.085	−0.297	0.313	0.044	0.087	−0.202
PCC	**0.655**	0.110	−0.442	0.101	0.308	−0.191
Concentration of Hg	0.326	0.306	0.205	**0.539**	0.141	**0.701**

Correlation coefficients > 0.5 are statistically significant (shown in bold, p ≤ 0.05).

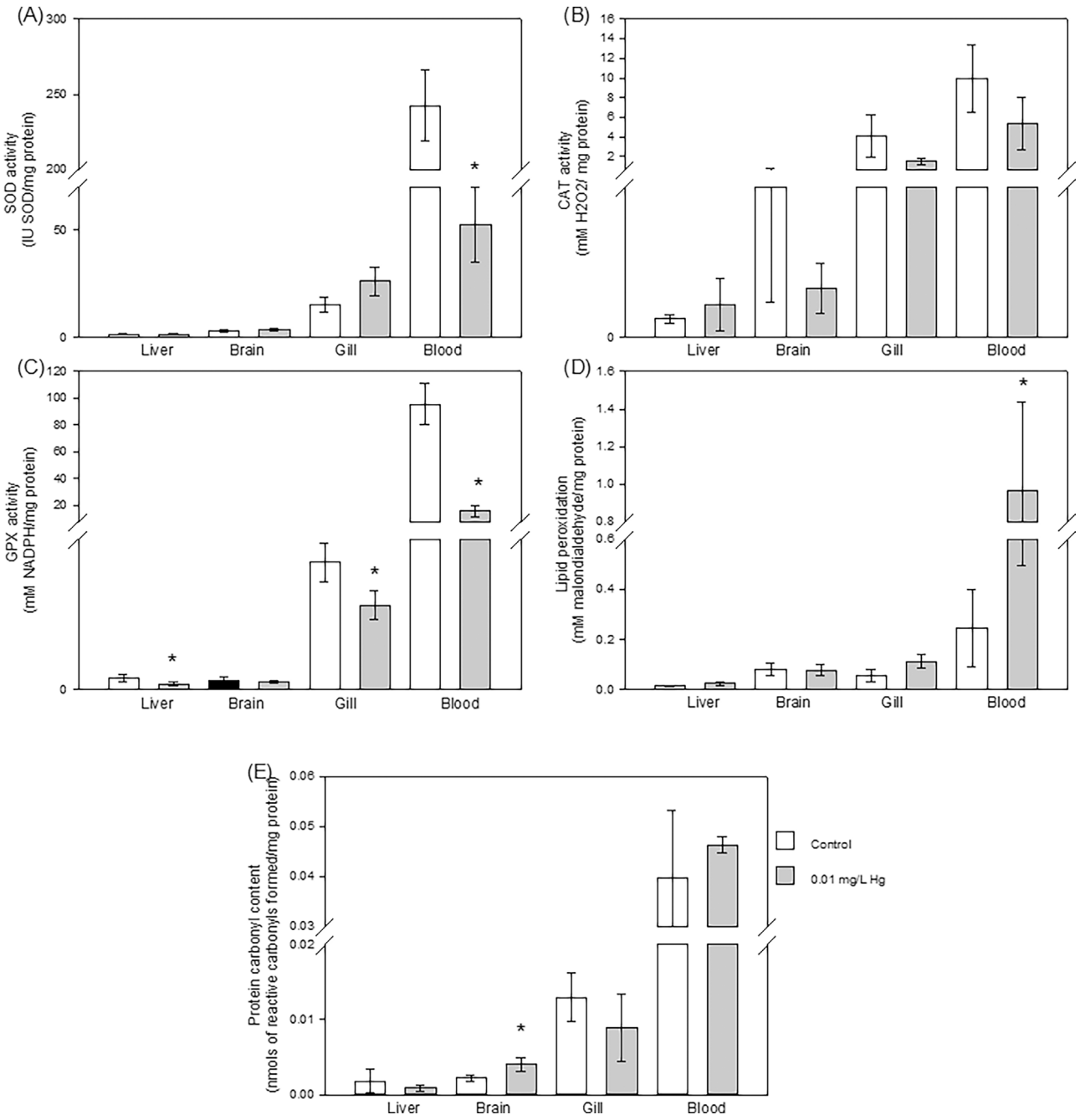

Fig. 1. Oxidative stress biomarkers: activity of the enzymes (A) superoxide dismutase, SOD; (B) catalase, CAT; and (C) glutathione peroxidase, GPx; as well as (D) lipid peroxidation, LPX; and (E) protein carbonyl content, PCC, in liver, brain, gill and blood of *Cyprinus carpio* exposed to 0.01 mg Hg/L for 96 h. Values are the mean of six replicates ± SEM. *Significantly different from the control group (*t*-test, $p < 0.05$).

4. Discussion

Exposure to $HgCl_2$ (0.01 mg Hg/L) for 96 h was sufficient to induce changes in antioxidant enzymes activity, but these changes were dependent on the tissue evaluated (Fig. 1A, B and C). A significant reduction in SOD activity relative to the control group was observed in blood, but no significant differences were found in other tissues. CAT activity showed nonsignificant reductions in brain, gill and blood and a nonsignificant increase in liver, while GPx activity decreased significantly in blood, liver and gill. A similar response has been reported in digestive gland of mussel exposed to 0.045 mg Hg/L (Verlecar et al., 2008). Since Hg, a redox-inert metal, does not undergo redox cycling, the damage induced on antioxidant defense systems is probably due to its capacity to bind to sulfhydryl groups of antioxidant enzymes (Guilherme et al., 2008). This decrease in enzyme activity can result in accumulation of ROS. This concurs with statements by Górska-Czekaj and Borucki (2013), who link Hg-induced oxidative stress to higher levels of H_2O_2. Furthermore, in studies on aquatic organisms, Hg has been found to reduce GSH levels in diverse organs, affecting enzymes that require this molecule, such as GPx and glutathione S-transferase (Verlecar et al., 2008; Cappello et al., 2016). Inhibition of antioxidant enzymes may favor the Haber-Weiss reaction as well as accumulation of oxyradicals that combine with H_2O_2 to form the hydroxyl radical (·OH) which in turn affects biomolecules such as lipids and proteins.

LPX is a major contributing factor of loss of cellular function under oxidative stress conditions (Verlecar et al., 2008; Monteiro et al., 2010). Since LPX is an indicator of oxidative damage in cell components, our results (Fig. 1D) suggest that ROS formation was induced by Hg,

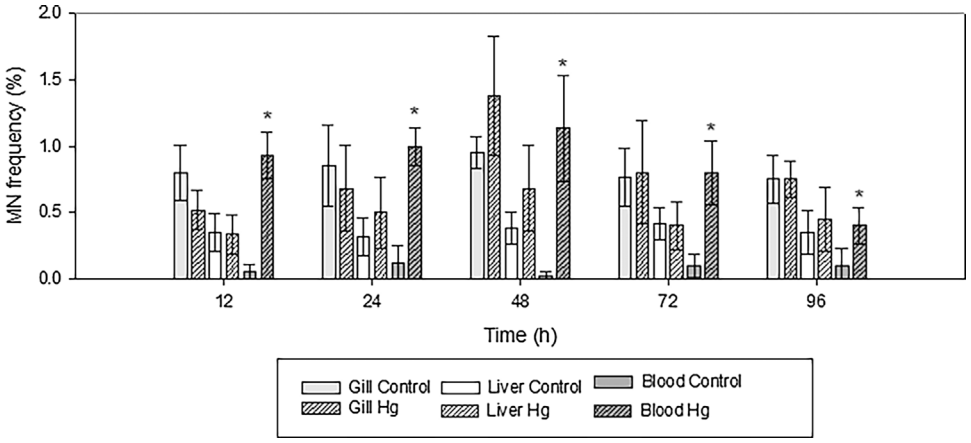

Fig. 2. Micronuclei (MNi) frequency in tissues of *Cyprinus carpio* exposed to 0.01 mg Hg/L for 12, 24, 48, 72 and 96 h. Values are the mean of six replicates ± SEM. *Significantly different from the control group (S-N-K *post hoc*, $p < 0.05$).

damaging to a greater extent blood, followed by liver and gill. This is consistent with reduced antioxidant enzyme activity, particularly GPx. In other fishes exposed to Hg, particularly Atlantic salmon (*S. salar*) and the South American freshwater fish *Brycon amazonicus*, LPX increases have been observed in liver, gill, brain, kidney, muscle and heart at concentrations of 10 and 100 mg Hg/kg, in diets supplemented with mercuric chloride and 0.15 mg Hg/L (Berntssen et al., 2003; Monteiro et al., 2010).

Protein oxidation detected through protein carbonyl (CO·) content can also be used as a biomarker of oxidative stress (Almroth et al., 2005; Parvez and Raisuddin, 2005). In particular, digestive gland of the bivalve *Perna viridis* and liver, muscle, gill and heart of *B. amazonicus* evidenced increases in CO· amounts with exposure to Hg (Verlecar et al., 2008; Monteiro et al., 2010). Fig. 1E shows that PCC increased significantly in brain only. Mercury ions induce oxidative stress through ROS production by breaking off the mitochondrial electron transport chain. This process may give rise to protein oxidation. In both mammals and fish, brain tissue has high levels of mitochondrial oxidative metabolism since it must meet the elevated demand for ATP required for neural processing, making it more susceptible to oxidation by biomolecules such as proteins (Berntssen et al., 2003) and eventually to protein degradation by proteases (Höhn et al., 2013).

Compounds derived from protein or lipid oxidation are toxic due to their carcinogenic and genotoxic potential. Their presence in different tissues is therefore of serious concern in terms of their potential toxic effects on the health of aquatic organisms (Valavanidis et al., 2006). Since DNA damage is one of the possible consequences of oxidative stress, it was decided to evaluate genotoxicity in tissues showing higher levels of oxidative damage, and at least one parameter with correlation value between oxidative damage biomarkers (LPX or PCC) and antioxidant defenses (SOD, CAT or GPx) greater than 0.9 (blood, gill and liver; Table 2).

As regards genotoxicity, Fig. 2 shows a tendency for MNi frequency to increase in gill cells and hepatocytes at early exposure times and decrease thereafter. This tendency for MNi to increase in both cell types leads to the assumption that most of these cells were in interphase or at the start of the cell cycle, and while both tissues have a high mitotic index in the presence of genotoxic agents, repair mechanisms also exist which can decrease MNi frequency (Çavaş, 2008). In contrast, a significant increase in MNi levels was observed at all exposure times in peripheral blood erythrocytes, with peak values at 48 h followed by a slight reduction, as well as a high correlation (0.701) between Hg concentration in the specimen and MNi frequency. These results are consistent with findings in the fish *Channa punctata* exposed to 0.081 mg Hg/L, in which an increase in MNi induction was followed by slight stabilization and gradual decline (Yadav and Trivedi, 2009). Other studies have found that inorganic Hg induces clastogenic and/or aneugenic lesions since it elicits an increase in the frequency of erythrocyte nuclear abnormalities as well as a high rate of DNA breaks in blood cells of the fishes *L. aurata* and *Dicentrarchus labrax* (Pereira et al., 2010; Mohmood et al., 2012).

As for damage evaluated by comet assay, Fig. 3 shows that DNA damage increased significantly in all tissues and cells exposed to Hg. Diverse hypotheses seek to explain the molecular mechanism of genotoxicity induced by exposure to inorganic and organic mercury compounds. These assumptions include direct interaction of Hg with DNA, free radical production and oxidative stress, inhibition of spindle formation (through the action of Hg on microtubules) and modifications in DNA repair mechanisms. These processes are not mutually exclusive; therefore, Hg can probably trigger every one of them, inducing

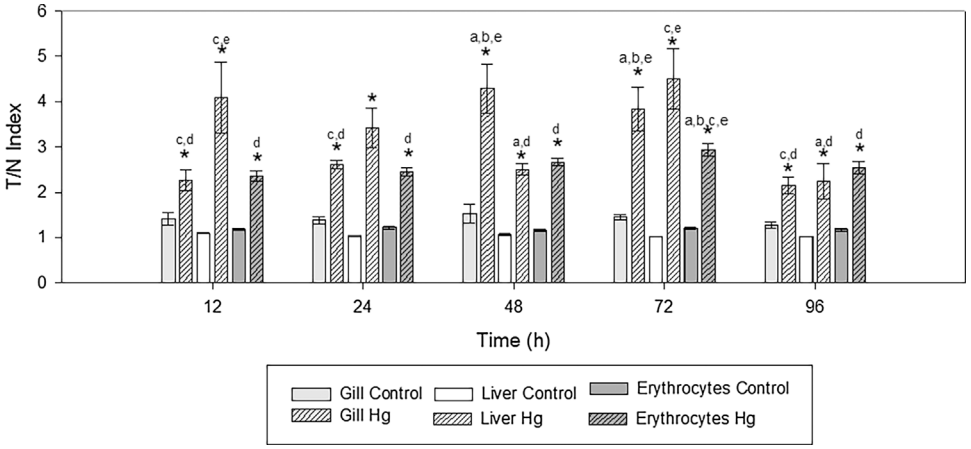

Fig. 3. Determination by comet assay of DNA damage in tissues of C. carpio exposed to 0.01 mg Hg/L for 12, 24, 48, 72 and 96 h. Values are the mean of six replicates ± SEM. Significantly different from: *control group; [a]12 h; [b]24 h; [c]48 h; [d]72 h [e]96 h. (S-N-K *post hoc*, $p < 0.05$).

genotoxicity on exposed populations (Crespo-López et al., 2009).

Response in the biomarkers evaluated shows that the tissues tested differ as to degree of susceptibility; e.g. gill and blood are more sensitive to oxidative stress since they show signs of redox homeostasis insufficiency, while blood were more susceptible to genotoxicity than other tissues evaluated. These data suggest that tissues differ in terms of antioxidant capacity, rate of ROS formation, and DNA damage intensity. The gills and certain blood cells such as erythrocytes are usually the main target organs of diverse contaminants (Val, 2000; Monteiro et al., 2010). The gills are recognized as the major route of Hg uptake, which induces deep lesions in lamellae, decreases permeability and the surface area for the exchange of gases between water and blood, and modifies homeostasis of body systems (Berntssen et al., 2003; Jakka et al., 2007; Monteiro et al., 2010). The gills are also a pathway of Hg detoxification. Naïja et al. (2016) found that chloride cell hypertrophy is produced by the need for gills to eject uptaken Hg, and that reduced Hg burden in the gills was accompanied by increased gene expression of antioxidant proteins (SOD and GPx) as well as metallothioneins, protecting the gills from Hg accumulation, LPX and structural damage to tissues. This is consistent with our correlation analysis results (Table 3) in which an r value > 0.5 was found for Hg concentration and MNi frequency. Probably, accumulation of the metal induces major changes in tissues, with a consequent increase in chromosome damage.

Once Hg is uptaken, it enters in contact with blood components, and this metal is known to have a high affinity for biomolecules rich in sulfhydryl groups such as albumin. Also, Gailer (2007) states that Hg has a high rate of diffusion across the cell membrane (4 min), subsequently forming a complex – GS-Hg-Hgb – with intracellular GSH and hemoglobin. On the other hand, since Hg has a high affinity for molecules like oxygen (O_2), binding of O_2 to sulfhydryl groups favors methemoglobin formation (Ribarov et al., 1984; Lushchak, 2008). This may explain the high susceptibility to oxidative damage observed in erythrocytes of exposed fish in our study as well as the strong correlation between DNA damage assessed by comet assay and SOD and CAT activity (Table 3). Finally, as a result of the oxidative stress induced by Hg on erythrocytes, the body needs to produce large numbers of functional cells, so that changes in the genetic material of circulating erythrocytes provide early signs of the existence of lesions in hematopoietic tissues (Elahee and Bhagwant, 2007).

On the other hand, while liver showed lower levels of oxidative damage and MNi frequency in our study, DNA breaks did increase with Hg exposure. Similar increases have been found in other fish species exposed to metals or living in metal-contaminated sites (Kaur and Dua, 2016). However, unlike the other tissues evaluated, in our study, a high correlation was found between antioxidant enzyme activity and DNA damage, but not with Hg levels in specimens (Table 3). This can be taken as a sign of the presence of different thresholds for expression of toxicity, either in the form of enzymatic inhibition or as DNA damage (Guilherme et al., 2012). The observed tissue-specific responses may also be due to the distinctive physiological activities of the organs evaluated with regard to activation or detoxification of particular pollutants, or repair of different types of DNA strand breaks (Della Torre et al., 2010; De la Torre et al., 2005; Monteiro et al., 2010).

Bioaccumulation of toxic metals in fish tissues depends on the uptake and elimination rates of individual metals, their bioavailability, and environmental characteristics among others (Fatima et al., 2014; Monteiro et al., 2010). Like other metals, Hg varies in its affinity to fish tissues; it is usually accumulated mainly in the liver, kidneys and gills (Mela et al., 2007; Monteiro et al., 2010). Although Hg was not detected in all organs evaluated, Hg concentration at 96 h in carp as well as water from exposure systems was determined, the BCF obtained being 0.7168 ± 0.0861. Hg has been said to have a high BCF (4.95 on average), but these reports come mostly from chronic studies (McGeer et al., 2003) or exposure to methylmercury, the most liposoluble and toxic form of this metal (Berntssen et al., 2003).

5. Conclusions

The present study establishes that oxidative stress and genotoxicity were induced by Hg on specimens exposed to a concentration equal to the maximum permissible limit in Mexico for aquatic life protection, since inhibition of antioxidant enzymes activity and increased LPX and DNA damage occurred. Blood and gill in particular were more sensitive to oxidative stress compared to liver or brain, while erythrocytes were more sensitive to genotoxicity than gill cells or hepatocytes. Since the functions performed by these organs are essential for the survival and growth of living organisms, the presence of Hg in aquatic ecosystems at permissible concentrations (0.01 mg/L) may have adverse effects on wild populations of *Cyprinus carpio*. Current limits should therefore be reviewed and, where appropriate, corrected.

Conflict of interest

The authors declare that the results contained in this paper involve no conflict of interests.

Ethical approval

All applicable international, national and institutional guidelines for the care and use of animals were followed. All procedures were performed in accordance with the ethical standards of the institution where the studies were conducted.

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