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Environmentally relevant concentrations of glibenclamide induce oxidative stress in common carp (*Cyprinus carpio*)

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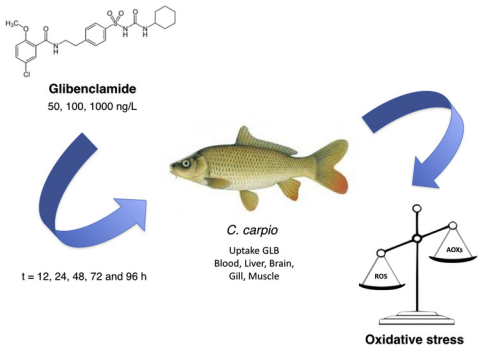
HIGHLIGHTS

- GLB is antidiabetic drug used around the world.
- GLB is released into water bodies.
- This work evaluated GLB-induced oxidative damage in *Cyprinus carpio* .
- GLB uptake is high in liver and muscle of *C. carpio* and lower in brain and gill.
- Exposure to GLB induced oxidative stress in *C. carpio*.
- Exposure to ng/L of GLB induces oxidative damage in fish.

GRAPHICAL ABSTRACT



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ABSTRACT

The hypoglycemic pharmaceutical glibenclamide (GLB) is widely used around the world. This medication is released into the environment by municipal, hospital and industrial wastewater discharges. Although there are reports of its environmental occurrence in the scientific literature, toxicity studies on aquatic species of commercial interest such as the common carp *Cyprinus carpio* are scarce. The present study aimed to evaluate the oxidative stress induced on *C. carpio* by environmentally relevant concentrations of GLB. Biomarkers of oxidative damage such as hydroperoxide content, lipid peroxidation and protein carbonyl content were evaluated as well as the activity of the antioxidant enzymes superoxide dismutase and catalase. The concentration of GLB was determined in water as well as in gill, liver, muscle, brain and blood of carp at 12, 24, 48, 72 and 96 h. The findings obtained in the study prove that GLB induces increases in biomarkers of oxidative damage and antioxidant enzyme activity in the teleost *C. carpio*, that

Abbreviations: CAT, Catalase; CHP, Cumene hydroperoxide; CYP450, Cytochrome P450; EC50, Median effective concentration; GLB, Glibenclamide; HPC, Hydroperoxide content; HPLC-MS/MS, High performance liquid chromatography-tandem mass spectrometry; LC50, Median lethal concentration; LPX, Lipid peroxidation; MDA, Malondialdehyde; MEC, Molar extinction coefficient; OD, Optical density; PCC, Protein carbonyl content; SOD, Superoxide dismutase; TBARs, Thiobarbituric acid reactive substances.

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this response is not concentration dependent and that the organs evaluated bioconcentrate this hypoglycemic agent. These findings permit us to conclude that the presence of GLB in water bodies represents a risk for aquatic species.

1. Introduction

Type 2 diabetes mellitus is one of the metabolic disorders currently prevalent at world level. In 2012, the International Diabetes Federation estimated that more than 371 million people live with this disease and 4.8 million die from it each year. At the global level, it is estimated that by 2030 the number of diabetic persons will have increased to 439 million, representing 7.7% of the adult population (aged 20–79 years) in the world (Juan et al., 2013).

The group of pharmaceuticals used to treat this ailment are oral hypoglycemic agents. One of the more commonly prescribed of these is glibenclamide (GLB) which is widely produced and consumed throughout the world, it is the most common treatment for gestational diabetes since 2007 in US and their use increased from 7.4% to 64.5% in this country (Balsells et al., 2015; Castillo et al., 2014) Previous studies have determined the presence of GLB in diverse effluents: in Spain in the municipal wastewater of Rubí (Barcelona) at concentrations of 19.2–100 ng L^{-1} (Radjenovic et al., 2007; Verlicchi et al., 2012); in wastewater and sewage sludge of Terrassa (Barcelona) at 15900 ng L^{-1} (Radjenovic et al., 2009); in Río Ebro water at concentrations of 40 ng L^{-1} to 2000 ng L^{-1} (Postigo Rebollo et al., 2011); and in water treatment plants of Catalonia at concentrations of 0.6 and 4.6 ng L^{-1} (Ginebreda et al., 2012, 2015). The presence of GLB has also been detected in reclaimed water for irrigation in Florida (US) at concentrations of 22 ng L^{-1} (Wang and Gardinali, 2013) and surface waters in Portugal and Spain at 10.9–43.6 ng L^{-1} (Osorio et al., 2016).

As regards GLB toxicity, it has been proved to have harmful effects on humans, rodents and other animal species like DNA damage, oxidative stress, genotoxicity and mutagenesis (Ibarra-Costilla et al., 2010; Martínez-Pérez, 2004; Morales-Alvarado and Murga-Valdez, 2007; Nazaroglu et al., 2009; Tüzün et al., 1999). However, very little is known about its toxic effects on hydrobionts common in aquatic systems or its behavior and fate in the environment (Barceló et al., 2009; Gros et al., 2010; Richardson and Terners, 2009, 2011; Santos et al., 2013).

The toxokinetics of GLB in mammals includes rapid oral absorption; GLB binds to plasma proteins in a high percentage. Also, it is biotransformed in the liver via hydroxylation of the cyclohexyl ring at the cis-3 and trans-4 prositions by the cytochrome P450 (CYP450) system, in which the isoforms 2C9, 2C19 and 3A4 are involved. GLB is also biotransformed in the kidneys although this is a minor process. This pharmaceutical is eliminated from the body through renal and biliary excretion (Aguilar-Bryan et al., 1995; Philipson and Steiner, 1995). Although the presence of the 2C9 isoform has not been reported in the international literature, the same events may occur in fish.

In the phase I biotransformation of GLB, a complex called oxycytochrome P450 (O_2-P450-Fe^{2+}-GLB) is formed. This complex breaks down, releasing the superoxide anion radical ($O_2^{\bullet}$), with regeneration of the ferric protein (P450- Fe^{3+}-GLB) and subsequent formation of H_2O_2. The latter products, called reactive oxygen species (ROS), are responsible for oxidative stress induction, eliciting damage in biomolecules such as lipids, proteins and nucleic acids (Guengerich, 2012; Krest et al., 2013).

The regulated production of free radicals and the maintenance of redox homeostasis are essential for the physiological health of organisms. The generation of ROS is induced by internal and external agents, such as phagocytes, enzymes (such as CYP450), radiation and exogenous chemicals. In the same way, the generation of ROS can be reduced or reversed by various enzymes, called antioxidants, such as superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) (Kovacic and Jacintho, 2001). The endogenous ROS serve as a second messenger in signal transduction and are thought to be important in ion transport, immune defense of the host, transcription and cell apoptosis (Lander, 1997; Dennery, 2007). However, ROS can also be harmful due to their covalent or irreversible binding to cellular macromolecules, they are responsible for diverse effects on cells and tissues associated with mutagenesis and carcinogenesis (Ibarra-Costilla, 2004; Martínez-Pérez, 2004).

Oxidative stress refers to a state in which the cell is exposed to an increasingly oxidizing environment and antioxidant defense mechanisms are overcome, so that cellular redox status is altered; this is considered one of the major mechanisms of action of toxic substances due to this causes the irreversible oxidation of DNA, proteins and lipids, which leads to the inactivation of many enzymes and cell death and it can also affect gene expression by interfering with the activity of redox-sensitive transcription factors and signal transduction by the oxidation of thiols (Martínez, 2005; Sahambi and Hales, 2006). The oxidative damage induced on lipids, proteins and nucleic acids, and its effects on enzymatic mechanisms of antioxidant defense in living organisms have been used in recent years as a biomarker for monitoring environmental contamination (Dalle-Donne et al., 2003; Kadiiska et al., 2005; Lam and Gray, 2003).

Fish toxicity studies are among the most effective methods for understanding the harmful effects induced by environmental contaminants in aquatic systems. Fish play a major role in aquatic food webs, where they usually occupy an intermediate or high position: they not only feed on a variety of aquatic predators, they are also an important source of food for human populations around the world (van der Oost et al., 2003).

To evaluate the toxic effects of contaminants in diverse water bodies, test species known as bioindicators are used. The common carp *Cyprinus carpio* is frequently used as a bioindicator species, since cyprinids are one of the major groups of teleost fishes cultured for commercial purposes throughout the world and its members are also highly sensitive to toxic substances, show rapid response to changes in their aquatic environment and are easy to maintain in the laboratory (Elizalde-Velazquez et al., 2016; Gómez-Oliván et al., 2014; Huang et al., 2007; Islas-Flores et al., 2013, 2014; San Juan-Reyes et al., 2013, 2015). While some studies have shown that GLB induces oxidative stress in mammals such as male albino rat neonates at doses of 5 mg/kg weight (Tüzün et al., 1999) and changes in the activity of the enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase in male Sprague Dawley rat at the same dose (Nazaroglu et al., 2009), the scientific literature includes no studies on the capacity of GLB to induce oxidative stress on aquatic species of commercial interest such as common carp. Therefore, the present study aimed to evaluate the toxicity induced by three environmentally relevant concentrations of GLB (50, 100

and 1000 ng L^{-1}), which were selected based on available data of occurrence of GLB in diverse water bodies. Brain, gill, blood, liver and muscle of *C. carpio* were analyzed to evaluate oxidative stress biomarkers such as hydroperoxide content (HPC), lipid peroxidation (LPX) and protein carbonyl content (PCC), as well as the activity of the antioxidant enzymes SOD and CAT.

2. Materials and methods

2.1. Test substance

Glibenclamide (CAS No. 10236-21-8, >99% purity) was purchased from Sigma-Aldrich (Toluca, State of Mexico). A stock solution was prepared with 1 g GLB in 0.02% DMSO for dilution in 1 L distilled water. From this primary solution all other GLB concentrations (50, 100 and 1000 ng L^{-1}) were prepared.

2.2. Species procurement and acclimation

Test organisms used in the study were obtained from the common carp aquaculture center in Tiacaque (State of Mexico). In the acute toxicity assay, one-month-old alevins of *Cyprinus carpio*, 2.5 ± 0.5 cm long and weighing 5.5 ± 0.5 g, were used. In the sublethal toxicity assay, three-month-old juveniles, 15.5 ± 0.5 cm long and weighing 57.5 ± 4.5 g, were used. Fish were securely placed in sealed polyethylene bags containing two-thirds water with 80–90% oxygen saturation, transported to the laboratory where they were stocked in a large 500-L tank filled with 450 L of dechlorinated tap water previously reconstituted with salts, and acclimated for 30 days prior to the experiment. During acclimation, carp were fed Pedregal Silver™ fish food and ¾ of the tank water was replaced every 24 h. The physicochemical characteristics of reconstituted water were maintained: temperature 20 ± 2 °C, oxygen concentration 80–90%, pH 7.5–8.0, total alkalinity 17.8 ± 7.3 mg L^{-1}, total hardness 18.7 ± 0.6 mg L^{-1}. A natural light/dark photoperiod was maintained (12:12 h).

Water from the fish farm was analyzed by atomic absorption to ensure that carp had not been exposed to other contaminants like As, Cd, Cu, Cr, Hg, Ni, Pb and Zn. The results obtained didn't show pharmaceuticals or other contaminants that might interfere with the experiment.

2.3. Determination of acute toxicity (median lethal concentration, LC_{50})

The procedure used to determine the LC_{50} was performed as provided in Guideline 203 of the Organization for Economic Cooperation and Development (OECD, 1992), for determination of acute toxicity in fish exposed to chemical substances. In this assay, five exposure systems with five different concentrations of GLB (132, 209, 332, 526 and 834 mg L^{-1}) were used, as well as a sixth GLB-free control system. Ten fish at the alevin stage were exposed in each system. As stipulated in Guideline 203, the experiment was static without medium renewal. The assay was performed in triplicate, using a total of 180 fish.

Test system containers (60 × 40 × 20-cm glass tanks) were added the aforementioned GLB concentrations. The systems were maintained at ambient temperature with a natural light/dark photoperiod (12:12 h); constant aeration (80–90% oxygen concentration) was provided and fish were not fed during the assay.

As recommended in the OECD Guideline, the exposure time was 96 h and test specimens were observed at 12, 24, 48, 72 and 96 h. The principal variable evaluated in this experiment was mortality. The 96-h LC_{50} of GLB and its 95% confidence limits ($p < .05$) were estimated using the EPA Probit analysis program v1.5. Also, this protocol was reviewed and approved by the Bioethics Committee of the Universidad Autonoma del Estado de Mexico (UAEM) to ensure that it was carried out in accordance with institutional standards for the care of animal test subjects. Provisions set out in the official Mexican norm on the production, care and use of laboratory animals (NOM-062-ZOO, 1999) were also taken into account.

2.4. Quantification of GLB by high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS)

2.4.1. Equipment and chromatographic conditions

Quantification of GLB in water and tissue samples was performed using an Agilent 1100 HPLC system (Palo Alto, CA) equipped with Agilent vacuum degasser, quaternary pump and autosampler. Separation was done with Agilent LiChrospher® 100 RP-18 columns (125 mm × 4 mm ID, 5 μm particle size). The injection volume was 30 μL; the flow rate was 0.7 mL min^{-1}. Separation was made by gradient elution using 0.1% formic acid (v/v) as solvent A and an aqueous solution of 10 mM ammonium formate [containing 0.1% formic acid (v/v)] as solvent B. The gradient elution program was: 0–3 min: 10% solvent A; 3–5 min: linear increase to 50% A; 3–8 min: linear increase to 90% A; 8–10min: hold at 90% A; 10–10.1 min: linear decrease to 10% A; with a final hold at 10% A until 12.0 min. The column was thermostated at 25 °C.

MS was performed with an Agilent 6520 Q-TOF unit equipped with electrospray ionization source. The settings used for GLB ionization were: MS capillary voltage 3800 V; drying-gas flow rate 12 L min^{-1}; drying-gas temperature 350 °C; nebulizer pressure 60 psi. The system operated in the high resolution mode (4 GHz). Mass axis was calibrated over the range 70–3200 m/z using the manufacturer-provided mixture. A sprayer with a reference solution was used for continuous calibration in positive ionization mode using as reference masses 121.05 m/z and 922.01 m/z. The optimization of spectrometer parameters for GLB was: retention time 8.56 min; precursor ion 494.15 m/z; product ions 169.0027 m/z (product ion used for quantification 171.0037 m/z); fragmentor 100 V; collision energy 10 eV; instrumental detection limit 0.20 ng L^{-1}.

2.4.2. Solid phase extraction

Chromabond Tetracycline cartridges were used for solid phase extraction of GLB from water and tissue samples. Cartridges were conditioned with 10 mL methanol, followed by 5 mL HPLC-grade water. Prior to extraction, the pH of water samples was adjusted to 8.5 by adding 0.1 M NaOH. Water samples (200 mL) were percolated through the cartridges at a flow rate of approximately 10 mL min^{-1}. Then, each sample bottle was rinsed with 10 mL HPLC-grade water and the rinse was drawn through the cartridge. Cartridges were washed with a further 5 mL HPLC-grade water and dried for 10 min. GLB was eluted using four successive aliquots of 1 mL methanol at a flow rate of approximately 1 mL min^{-1}. The eluates were collected in 10-mL collection tubes and evaporated at room temperature to dryness with a gentle nitrogen stream. Then, the extracts were reconstituted in 1 mL methanol.

Gill, liver, brain and muscle samples were lyophilized, and accelerated solid-phase extraction was employed using 1 g of lyophilized tissue, methanol with 1% acetic acid, and sand (20–30 mesh particle size) as the dispersing agent. Fiberglass and cellulose filters were used to retain the lipidic phase. Extraction conditions were: 1000 psi; 25 °C; heat-up time 6 min; static cycle 3 × 10 min; flush volume 100%; purge time 60 s; 11 mL cells contained in one cellulose filter.

At the end of the procedure, 15 mL of the sample were obtained for subsequent analysis, under the conditions described in section 2.4.1.

2.5. Determination of oxidative stress

Three different concentrations of GLB (50, 100 and 1000 ng L^{-1}; hereafter referred to as C1, C2 and C3 respectively) were used for this assay. Exposure times were 12, 24, 48, 72 and 96 h. The experiment comprised three exposure systems (one for each test concentration) plus one GLB-free control system for each exposure time. The assay was performed in triplicate. Each system contained five fish at the juvenile stage (weight and size as described in section 2.2.). A total of 60 systems and 300 fish were used. The GLB concentrations used are based on relevant data of occurrence of GLB in diverse water systems, both municipal wastewater and river water, reported by Ginebreda et al. (2012, 2015), Postigo Rebollo et al. (2011), Radjenovic et al. (2007, 2009), and Wang and Gardinali (2013).

All test systems were prepared using water with the same characteristics and conditions described in section 2.2. The containers used were 120 × 80 × 40-cm glass tanks. Static systems without renewal were used. No food was provided to specimens and tank water was not changed during the assay. At the end of each exposure time, fish were anesthetized and blood was collected by puncture using a previously heparinized 1-mL hypodermic syringe. Puncture was made near the base of the caudal peduncle, at mid-height of the anal fin and ventral to the lateral line.

After puncture, fish were anesthetized in a container filled with water and 0.01% eugenol, and euthanized prior to removal of the gills, liver, muscle and brain. Then, 1 g of each organ were placed in phosphate buffer solution (pH 7.4), homogenized and then centrifuged at 12,500 × g and −4 °C for 15 min. Centrifuged samples were frozen and the supernatant was used to evaluate HPC, thiobarbituric acid reactive substances (TBARs), PCC, and SOD and CAT activity.

2.6. Determination of HPC

HPC determination was based on oxidation of ferrous ions to the ferric state due to presence of hydroperoxides under acidic conditions; ferric ions bind to the xylenol orange indicator to form a stable colored complex which is read at 560 nm, according to the method described by Jiang et al. (1992). First, 0.1 mL of supernatant was mixed with 0.9 mL reaction solution (0.25 mM $FeSO_4$, 25 mM H_2SO_4, 0.1 mM xylenol orange and 4 mM butylhydroxytoluene in 90% methanol v/v). The mixture was incubated for 60 min at ambient temperature while light protected, after which, absorbance was read at 560 nm against a blank subjected to the same conditions but using distilled water instead of supernatant. HPC content was estimated using the molar extinction coefficient (MEC) of $4.3 \times 10^4\ M^{-1}\ cm^{-1}$ and results were expressed as nM cumene hydroperoxide (CHP) mg^{-1} protein wet weight.

2.7. TBARs assay

Malondialdehyde (MDA) content was analyzed as TBARs by the spectrophotometric method described by Büege and Aust (1978). This procedure involved mixing 0.05 mL of supernatant and 0.45 mL Tris-HCl buffer solution (150 mM, pH 7.4). Next, 1 mL TBA-TCA reagent (0.375% thiobarbituric acid in 15% trichloroacetic acid) was added. The resulting solution was Vortex-shaken for 1 min, and samples thus prepared were immersed in a boiling water bath for 15 min, then immediately cooled at −5 °C for 3 min. Samples were later incubated for 30 min at 37 °C, then centrifuged at 3500 × g for 10 min. Absorbance was read at 535 nm using a blank subjected to the same treatment as samples, and distilled water instead of supernatant. Results were expressed as nM MDA, using the MEC of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ to calculate MDA content.

2.8. Determination of PCC

PCC was determined by the method described in Levine et al. (1994), as modified by Parvez and Raisuddin (2005) and Burcham (2007). In this analysis, supernatant (0.2 mL) was mixed with 0.15 mL 2,4-Dinitrophenylhydrazine (DNPH, 10 mM in 2 M HCl). The mixture was incubated for 60 min at room temperature while light protected, then supplemented with 0.5 mL of 20% TCA and centrifuged for 5 min at 11,000 × g. The resulting precipitate was collected and washed several times with 1:1 ethyl acetate:ethanol. Next, the bud was dissolved in a 1 mL guanidine solution (6 M, pH 2.3) and incubated at 37 °C for 30 min, then absorbance was read at 366 nm. Results were expressed as nM reactive carbonyls (C=O) mg protein wet $tissue^{-1}$, using the MEC of 21,000 $M^{-1}\ cm^{-1}$.

2.9. Determination of SOD activity

SOD activity was analyzed by the method of Marklund and Marklund (1974) as modified by Magnani et al. (2000). The supernatant was previously delipidized by adding 0.03 mL chloroform and 0.05 mL methanol, then Vortex-shaken for 1 min and centrifuged at 6000 × g for 15 min. Next, to 0.1 mL of delipidized supernatant was added 2.8 mL Tris-EDTA buffer solution, pH 8.2. The resulting mixture was shaken in the Vortex for 1 min, and was then added 0.05 mL of a 0.2 mM pyrogallol solution. The difference between optical density (OD) at 10 and 60 s, at 420 nm wavelength was determined. After this, the percentage of inhibition of pyrogallol autoxidation was obtained by dividing the difference in test sample ODs by the difference in control sample ODs and multiplying by 100. Results were expressed as IU SOD mg protein wet $tissue^{-1}$; to this end the quotient of the percentage of pyrogallol autooxidation was divided by 50%, since 1 IU SOD induces a 50% inhibition of pyrogallol autooxidation at 25 °C.

2.10. Determination of CAT activity

CAT activity was estimated by the Radi et al. (1991) method, which is based on evaluating enzyme activity when H_2O_2 is reduced in a medium prepared by mixing 1 mL isolation buffer solution (0.3 M sucrose, 1 mM EDTA, 5 mM HEPES and 5 mM KH_2PO_4) with 0.02 mL of supernatant, and supplementing with 0.2 mL of 20 mM H_2O_2. The CAT activity required to reduce the H_2O_2 substrate was measured at 0 and 60 s, at 240 nm, and was expressed as μM H_2O_2 mg protein wet $tissue^{-1}$. Values were calculated by substituting absorbance readings in the formula: CAT concentration = $(A_0 - A_{60})$/MEC, where the MEC of H_2O_2 is 0.043 $mM^{-1}\ cm^{-1}$. Results were expressed as units mg $protein^{-1}$; 1 unit is defined as the quantity of enzyme required to break down 1 μmol $H_2O_2\ min^{-1}$ at 25 °C and pH 7.0.

2.11. Determination of total protein content

Total protein content was determined by the spectrophotometric method described by Bradford (1976), in which 0.025 mL of supernatant is mixed with 0.075 mL deionized water and 2.5 mL Bradford reagent. This mixture was homogenized in a Vortex for 1 min and incubated for 5 min at room temperature while light protected, after which, absorbance was read at 595 nm. Absorbance readings were interpolated on a bovine serum albumin type curve and total protein content was expressed as mg protein wet tissue.

2.12. Statistical analysis

Oxidative stress results were statistically analyzed by one-way analysis of variance (ANOVA), and differences between means

were compared using the Bonferroni multiple comparisons test, with p set at <0.05. SPSS v9 software (SPSS, Chicago, IL) was used.

Pearson's correlation analysis was performed to find potential correlations between GLB concentrations in the organs evaluated, and the oxidative stress biomarkers used. To this end, Sigmastat v2.03 software was used.

3. Results

3.1. 96-h LC_{50}

The 96-h LC_{50} of GLB in alevins of *C. carpio* was 509 mg L^{-1}, with a 95% confidence interval of 505.96–512.16 mg L^{-1} ($p < .05$). The X^2 linear adjustment test was not significant at $p < .05$.

3.2. Quantification of GLB in water and tissue samples

Table 1 lists GLB concentration at the different exposure times in exposure system water and in the organs evaluated. As can be seen, beginning at 12 h, GLB concentration in water decreased in all exposure systems and increased gradually over time in all organs. GLB bioaccumulation was highest in blood, liver and muscle, and increased with time. Brain and gill were the organs of lowest bioaccumulation of GLB, although concentrations also increased in these organs with increasing exposure.

3.3. HPC

HPC results are shown in Fig. 1. At the C3 concentration (1000 ng L^{-1}), significant increases relative to the control group ($p < .05$) occurred from 24 to 96 h in ***brain*** (254% for 24 h, 207% for 48 h, 214% for 72 h and 119% for 96 h); from 12 to 96 h in ***blood*** (418% for 12 h, 614% for 24 h, 704% for 48 h, 669% for 72 h and 884% for 96 h) as well as ***liver*** (55% for 12 h, 94% for 24 h, 68% for 48 h, 66% for 72 h and 51% for 96 h); at 24 and 96 h in ***gill*** (37% for 24 h and 54% for 96 h); and from 12 to 96 h in ***muscle*** (368% for 12 h, 736% for 24 h, 734% for 48 h, 928% for 72 h and 803% for 96 h). At the C2 concentration (100 ng L^{-1}), significant increases were found from 12 to 96 h in ***blood*** (318% for 12 h, 314% for 24 h, 585% for 48 h, 598 for 72 h and 762% for 96 h) as well as ***liver*** (38% for 12 h, 46% for 24 h, 29% for 48 h, 32% for 72 h and 52% for 96 h); from 12 to 48 h in ***gill*** (26% for 12 h, 28% for 24 h and 26% for 48 h); from 24 to 96 h in ***brain*** (128% for 24 h, 84% for 48 h, 95% for 72 h and 115% for 96 h); and from 12 to 96 h in ***muscle*** (301% for 12 h, 450% for 24 h, 451% for 48 h, 436% for 72 h and 324% for 96 h). At the lowest concentration (C1, 50 ng L^{-1}), significant differences were observed only from 12 to 96 h in ***blood*** (318% for 12 h, 314% for 24 h, 585% for 48 h, 598% for 72 h and 762% for 96 h) as well as ***muscle*** (124% for 12 h, 379% for 24 h, 380% for 48 h, 394% for 72 h and 319% for 96 h).

3.4. TBARs assay

The MDA content induced by GLB is shown in Fig. 2. At the highest concentration (C3), significant increases with respect to the control group ($p < .05$) were observed from 12 to 96 h in ***blood*** (198, 315, 268, 211 and 171%), ***liver*** (148, 186, 195, 143 and 129%) and ***gill*** (312, 471, 437, 294 and 302%); from 72 to 96 h in ***brain*** (112 and 87%); and from 12 to 96 h in ***muscle*** (1391, 1621, 1309, 1334 and 850%). At the C2 concentration, significant increases were recorded from 12 to 96 h in ***blood*** (88, 169, 172, 177 and 111%); from 12 to 72 h in ***liver*** (150, 185, 167 and 92%); from 12 to 96 h in ***gill*** (137, 617, 409, 336 and 310%); from 72 to 96 h in ***brain*** (53 and 50%); and from 12 to 96 h in ***muscle*** (696, 835, 1223, 966 and 591%). At the lowest concentration (C1), significant differences were found from 24 to 48 h in ***blood*** (47 and 40%); from 48 to 96 h in ***liver*** (134, 145 and 147%); from 12 to 96 h in ***gill*** (103, 219, 141, 110 and 153%); from 72 to 96 h in ***brain*** (96 and 103%); and from 24 to 96 h in ***muscle*** (99, 346, 727 and 631%).

3.5. PCC

Fig. 3 shows PCC results. Significant increases with respect to the control group ($p < .05$) were observed at the C3 concentration from 12 to 96 h in ***blood*** (119, 108, 116, 100 and 119%); from 12 to 48 h in ***liver*** (47, 73 and 54%) as well as ***gill*** (47, 75 and 57%); and from 12 to 96 h in ***brain*** (233, 308, 322, 304 and 347%) and ***muscle*** (141, 231, 249, 401 and 675%). At the C2 concentration, significant increases were found from 12 to 96 h in ***brain*** (156, 218, 141, 216 and 299%) as well as ***muscle*** (111, 269, 234, 317 and 401%); and from 12 to 48 h in ***blood*** (67, 30 and 50%), ***liver*** (41, 49 and 30%) and ***gill*** (34, 75 and 26%). At the C1 concentration, significant increases occurred from 12 to 24 h in ***blood*** (28 and 24%) and ***liver*** (30 and 31%); from 24 to 48 h in ***gill*** (75 and 26%); from 24 to 96 h in ***brain*** (94, 212, 214 and 165%); and from 48 h to 96 h in ***muscle*** (90, 132 and 180%).

3.6. SOD activity

Results of SOD activity are shown in Fig. 4. Significant increases relative to the control group ($p < .05$) were observed at the C3 concentration from 24 to 72 h in ***blood*** (250, 90 and 37%); from 12 to 72 h in ***liver*** (194, 337, 310, 108 and 154%); from 12 to 48 h in ***gill*** (36, 55 and 59%) as well as ***brain*** (136, 114 and 73%); and from 12 to 96 h in ***muscle*** (399, 484, 505, 573 and 526%). At the C2 concentration, significant increases occurred from 12 to 72 h in ***blood*** (42, 169, 38 and 71%), ***liver*** (184, 302, 320 and 145%) and ***gill*** (52, 55, 38 and 44%); from 12 to 48 h in ***brain*** (68, 80 and 95%); and from 12 to 96 h in ***muscle*** (399, 484, 505, 537 and 526%). At the C1 concentration, significant increases were found from 12 to 48 h in ***blood*** (79, 147 and 118%); from 12 to 72 h in ***liver*** (95, 253, 357 and 232%); from 12 to 24 h in ***gill*** (57 and 31%); from 24 to 72 h in ***brain*** (51, 185 and 241%); and from 12 to 96 h in ***muscle*** (114, 159, 160, 1154 and 837%).

3.7. CAT activity

Fig. 5 shows the results of CAT antioxidant activity. Significant increases relative to the control group ($p < .05$) occurred at the C3 concentration from 12 to 96 h in ***blood*** (199, 393, 481, 278 and 11%) as well as in ***liver***, with even higher increases (404, 614, 738, 1144 and 446%), and also in ***gill*** (81, 92, 228, 343 and 103%), ***brain*** (264, 303, 675, 557 and 569%) and ***muscle*** (69, 279, 204, 122 and 119%). At the C2 concentration, significant increases were observed from 12 to 72 h in ***blood*** (154, 196, 253 and 49%) and ***liver*** (181, 259, 345 and 127%); from 24 to 72 h in ***gill*** (56, 135 and 319%); from 12 to 96 h in ***brain*** (248, 237, 147, 348 and 472%); and from 24 to 72 h in ***muscle*** (177, 78 and 84%). At the C1 concentration, significant increases occurred from 24 to 72 h in ***blood*** (27, 24 and 26%); from 24 to 48 h in ***liver*** (83 and 100%); from 24 to 72 h in ***gill*** (49, 74 and 100%); from 24 to 96 h in ***brain*** (116, 83, 197 and 347%); and from 24 to 48 h in ***muscle*** (35 and 45%).

3.8. Pearson's correlation analysis

Table 2 shows the correlations found between oxidative stress biomarkers, exposure times and GLB bioconcentration in organs. Values in bold indicate a closer correlation between analyzed variables.

Table 3 shows the bioconcentration values obtained from the ratio of the GLB concentration of each tissue between the concentration in water.

Table 1
Glibenclamide concentrations in exposure system water and evaluated organs.

Test concentration	Exposure time (h)	Water	Blood	Liver	Gill	Brain	Muscle
50 ng L^{-1} (C1)	12	38 ± 1.2	3.6 ± 0.8	2.8 ± 0.6	1.6 ± 0.4	0.9 ± 0.1	2.6 ± 0.5
	24	35 ± 1.3	3.8 ± 0.7	3.0 ± 0.7	2.1 ± 0.6	1.0 ± 0.2	2.8 ± 0.4
	48	31 ± 1.8	4.0 ± 0.8	3.2 ± 0.6	2.4 ± 0.7	1.4 ± 0.3	3.2 ± 0.6
	72	27 ± 1.1	4.8 ± 0.6	3.5 ± 0.8	2.8 ± 0.6	1.7 ± 0.6	3.5 ± 0.7
	96	20 ± 1.5	5.0 ± 0.7	3.8 ± 0.6	3.2 ± 0.5	2.1 ± 0.7	3.8 ± 0.6
100 ng L^{-1} (C2)	12	75 ± 1.8	5.2 ± 1.1	4.1 ± 1.1	3.8 ± 0.8	2.6 ± 0.7	4.2 ± 0.8
	24	70 ± 2.1	5.7 ± 1.3	4.4 ± 0.9	4.1 ± 0.9	3.2 ± 0.6	4.6 ± 0.7
	48	66 ± 1.3	6.2 ± 0.9	4.9 ± 0.8	4.5 ± 0.8	3.8 ± 0.9	4.8 ± 0.8
	72	52 ± 1.6	6.8 ± 1.2	5.3 ± 1.2	5.1 ± 0.9	4.1 ± 1.1	5.2 ± 0.9
	96	49 ± 2.1	7.1 ± 0.9	6.1 ± 1.0	5.8 ± 1.0	4.6 ± 0.9	6.1 ± 1.1
1000 ng L^{-1} (C3)	12	770 ± 3.8	45 ± 1.1	32 ± 2.1	26 ± 1.2	19 ± 1.1	29 ± 0.9
	24	623 ± 2.8	53 ± 2.3	37 ± 1.3	32 ± 1.0	23 ± 1.6	33 ± 1.1
	48	556 ± 3.1	59 ± 1.8	41 ± 1.6	38 ± 1.3	29 ± 2.1	38 ± 1.4
	72	447 ± 1.8	64 ± 1.7	47 ± 1.9	44 ± 1.6	31 ± 1.7	41 ± 1.0
	96	382 ± 2.1	69 ± 1.7	51 ± 1.5	47 ± 2.1	39 ± 1.8	47 ± 0.9

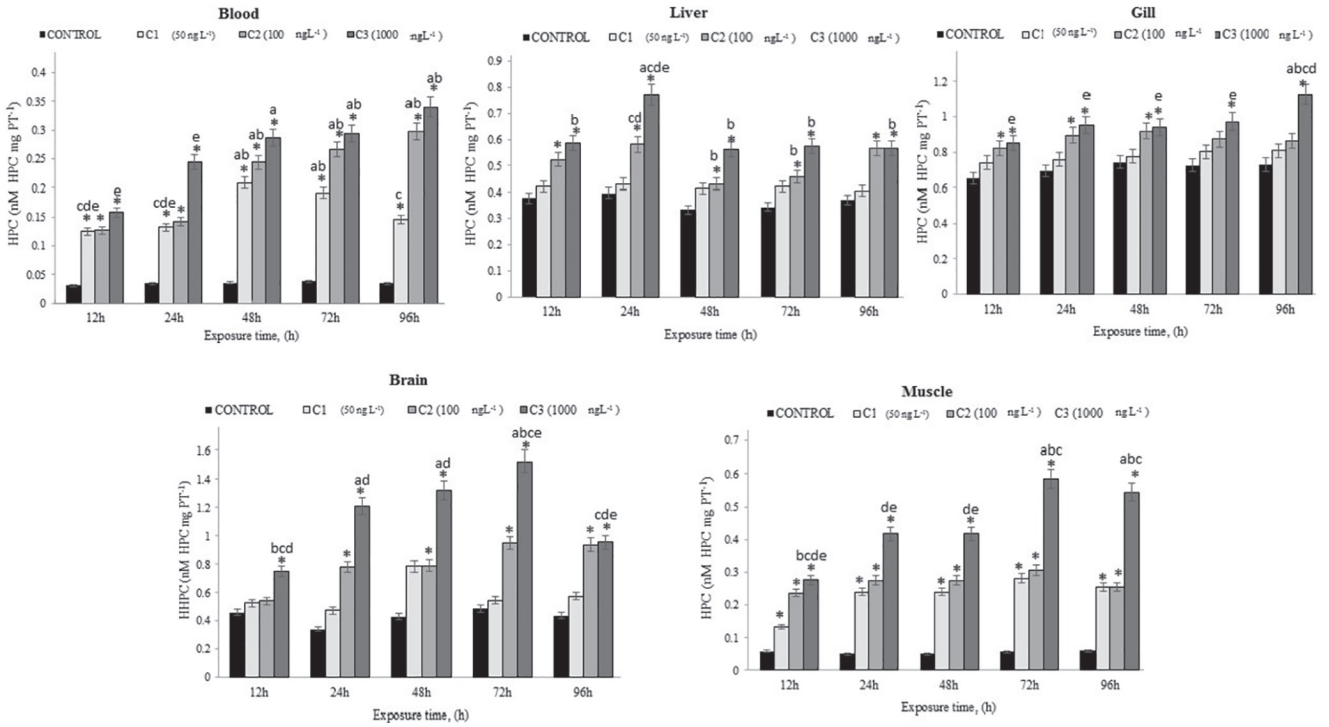

Fig. 1. Hydroperoxide content (HPC) in blood, liver, gill, brain and muscle of *C. carpio* exposed to three different concentrations of glibenclamide (C1, C2 and C3) for 12, 24, 48, 72 and 96 h. Values are the mean of three replicates ± SE. CHP = cumene hydroperoxide. Significant differences relative to: * control group; [a] 12 h; [b] 24; [c] 48 h; [d] 72 h; [e] 96 h; ANOVA and Bonferroni ($p < .05$).

4. Discussion

The present study is relevant and innovative due to the fact that the hypoglycemic agent GLB is widely used around the world and is released into the environment through municipal, hospital and industrial discharges. While the scientific literature includes studies on the occurrence of this pharmaceutical in the environment, there are very few studies regarding its toxicity. Existing ones have been conducted only on certain fish species as well as the crustacean *Daphnia magna*, and are aimed at determining the acute toxicity of this compound through LC_{50} determination. Therefore, the toxic effects induced by GLB on other fish species of commercial interest, such as *C. carpio*, need to be characterized using biomarkers that provide information on how this compound damages biomolecules such as proteins, lipids and nucleic acids. Results obtained in the present study determine that GLB is toxic. Although its LC_{50} values are high in comparison to other emerging contaminants, our study proves that concentrations of ng L^{-1} can induce lipid and protein oxidation as well as oxidative stress.

The LC_{50} of GLB was determined to be > 500 mg L^{-1} (505.96–512.16 mg L^{-1}) in our study. The pertinent OECD document on testing and assessment (OECD, 2001) ranks substances with an LC_{50} > 100 mg/L as category IV, which implies that GLB is not toxic to *C. carpio*.

Comparison of the LC_{50} of GLB in *C. carpio* and other species reveals that the common carp is a more resistant species. The safety data sheet issued in accordance with (EC) Regulation No. 1907/2006 as amended by (EC) No. 1272/2008 of the European Commission (06, 2013) states that the LC_{50} of GLB in *D. magna* and in fish is > 100 mg L^{-1}. Also, Cunningham et al. (2006) reported low GLB-

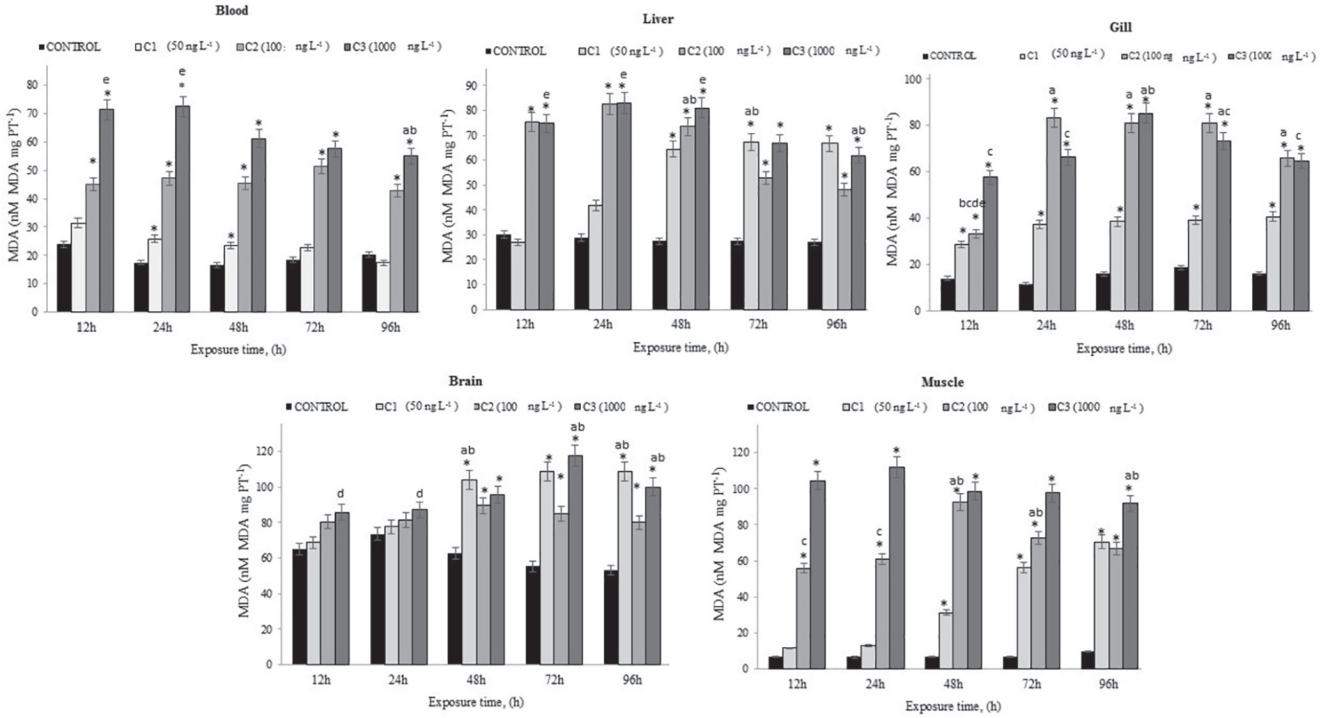

Fig. 2. Lipid peroxidation (LPX) in blood, liver gill, brain and muscle of *C. carpio* exposed to three different concentrations of glibenclamide (C1, C2 and C3) for 12, 24, 48, 72 and 96 h. Values are the mean of three replicates ± SE. MDA = malondialdehyde. Significant differences relative to: * control group; [a] 12 h; [b] 24; [c] 48 h; [d] 72 h; [e] 96 h; ANOVA and Bonferroni ($p < .05$).

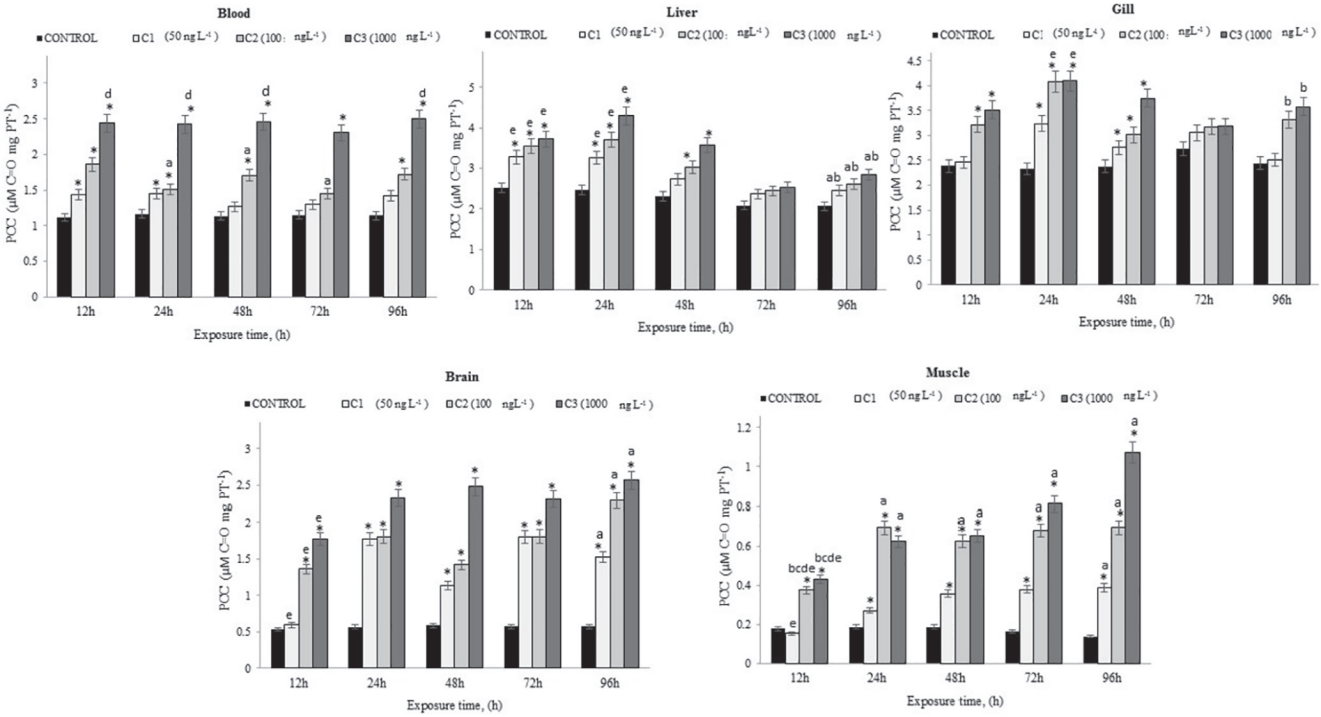

Fig. 3. Protein carbonyl content (PCC) in blood, liver, gill, brain and muscle of *C. carpio* exposed to three different concentrations of glibenclamide (C1, C2 and C3) for 12, 24, 48, 72 and 96 h. Values are the mean of three replicates ± SE. Significant differences relative to: * control group; [a] 12 h; [b] 24; [c] 48 h; [d] 72 h; [e] 96 h; ANOVA and Bonferroni ($p < .05$).

induced ecotoxicity; they found median effective concentration (EC_{50}) values > 100 mg L^{-1} in diverse species including daphnids, algae and fish. Fish mortality in the present study may be due to the fact that excess GLB induces a hypoglycemic state in fish, altering ion homeostasis. The latter process induces cell membrane depolarization and massive release of brain neurotransmitters in carp,

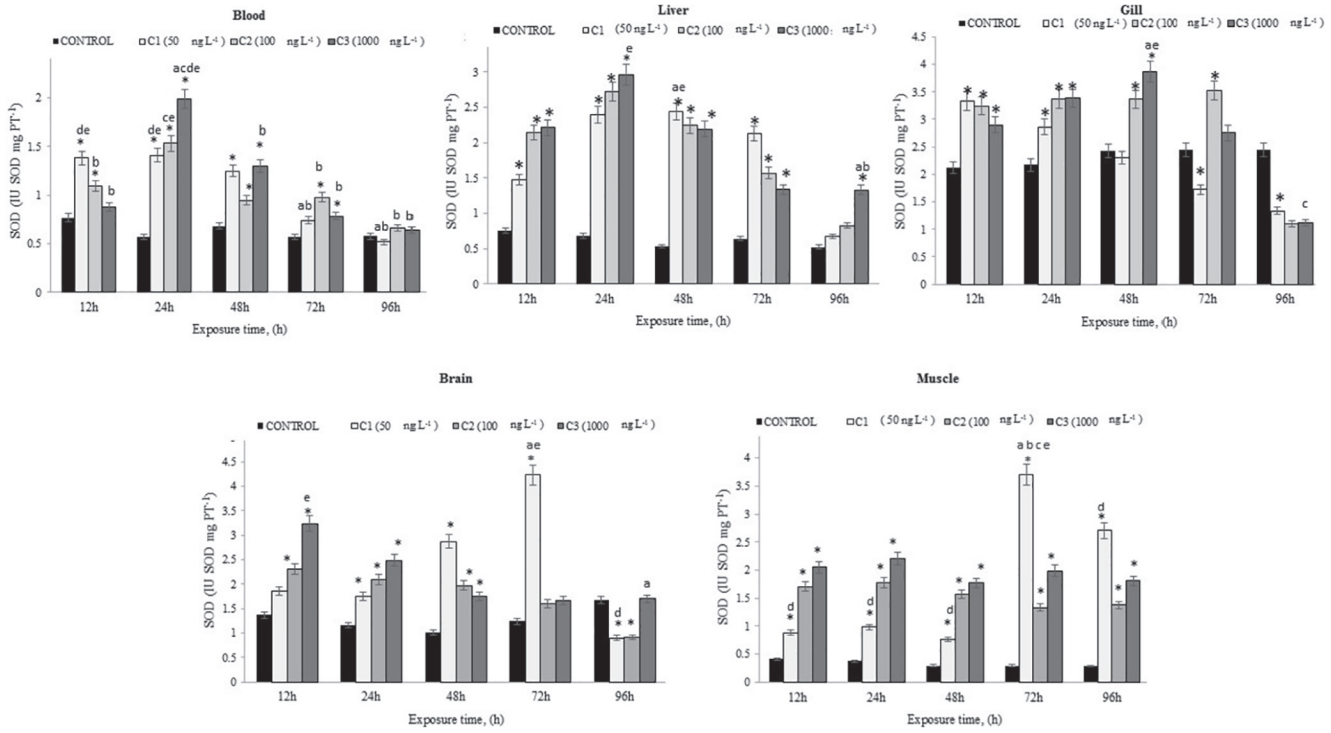

Fig. 4. Superoxide dismutase (SOD) activity in blood, liver, gill, brain and muscle of *C. carpio* exposed to three different concentrations of glibenclamide (C1, C2 and C3) for 12, 24, 48, 72 and 96 h. Values are the mean of three replicates ± SE. Significant differences relative to: * control group; [a] 12 h; [b] 24; [c] 48 h; [d] 72 h; [e] 96 h; ANOVA and Bonferroni ($p < .05$).

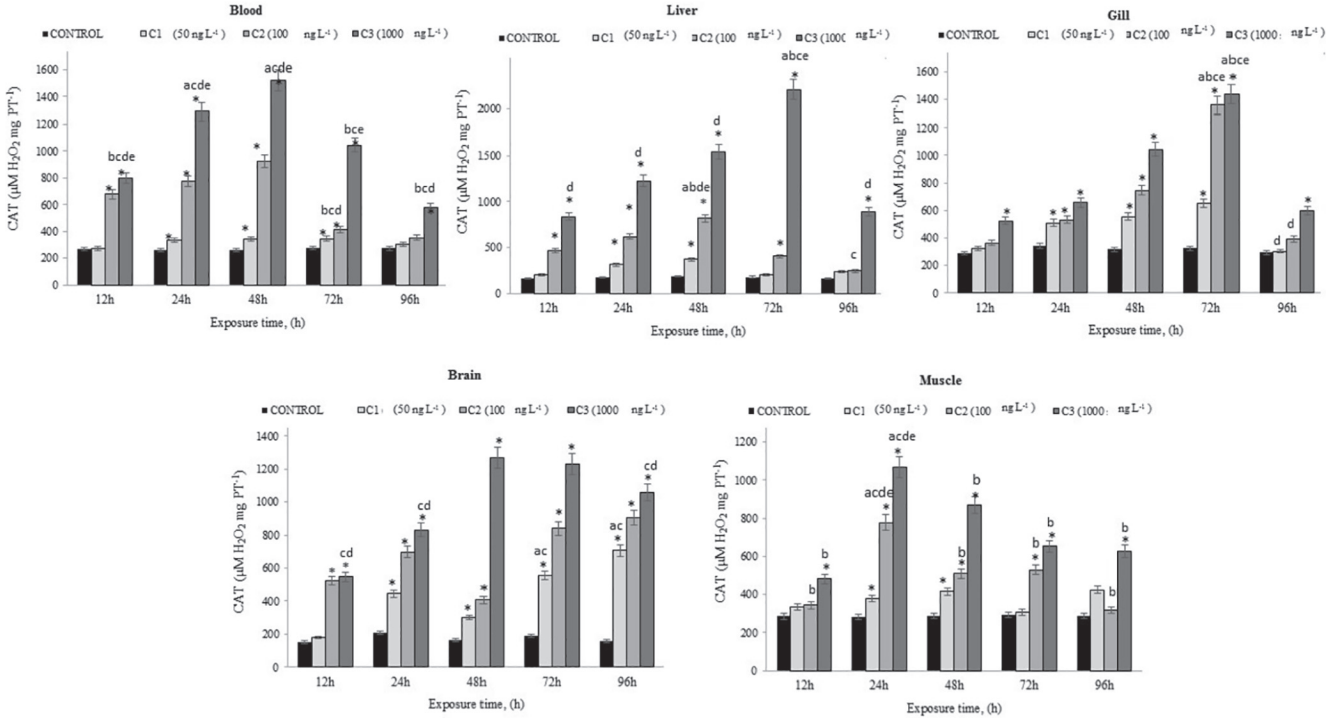

Fig. 5. Catalase (CAT) activity in blood, liver, gill, brain and muscle of *C. carpio* exposed to three different concentrations of glibenclamide (C1, C2 and C3) for 12, 24, 48, 72 and 96 h. Values are the mean of three replicates ± SE. Significant differences relative to: * control group; [a] 12 h; [b] 24; [c] 48 h; [d] 72 h; [e] 96 h; ANOVA and Bonferroni ($p < .05$).

including glutamate (Erecinska and Silver, 1989; Siesjo, 1988). Glutamate accumulation leads to an increase in mitochondrial calcium levels and ROS (Choi, 1987; Coyle and Puttfarcken, 1993) which induces oxidative stress. This contributes to occurrence of cerebral ischemia and hypoxia which inhibit the electron transport chain with loss of mitochondrial membrane potential and subsequent inhibition of ATP synthesis (Siesjo et al., 1995). The latter series of events may have contributed to fish mortality in our study.

Table 2
Pearson's correlation analysis of glibenclamide concentration in evaluated organs and oxidative stress biomarker results.

Biomarkers	Organs	Time (h)	Concentration			Biomarkers	Organs	Time (h)	Concentration		
			50 ng L^{-1}	100 ng L^{-1}	1000 ng L^{-1}				50 ng L^{-1}	100 ng L^{-1}	1000 ng L^{-1}
OXIDATIVE DAMAGE											
HPX	BLOOD	12	**0.801**	−0.295	**0.999**	**LPX**	BLOOD	12	**−0.564**	0.462	**0.644**
		24	0.347	−0.364	0.333			24	**−0.992**	**0.992**	**0.928**
		48	**−0.985**	**−0.740**	**0.788**			48	−0.289	**−0.891**	−0.123
		72	0.109	**−0.683**	**−0.687**			72	**−0.739**	**0.794**	**−0.821**
		96	**0.645**	0.235	**−0.980**			96	0.099	0.090	0.127
	LIVER	12	**−0.774**	**−0.990**	**0.998**		LIVER	12	0.165	**−0.854**	**−0.554**
		24	**0.793**	0.226	**0.914**			24	**0.987**	0.333	−0.059
		48	−0.148	**−0.860**	**0.887**			48	**0.961**	−0.421	**−0.925**
		72	0.012	**0.510**	**0.573**			72	−0.218	−0.319	**−0.725**
		96	**−0.905**	**−0.650**	**0.981**			96	**0.592**	**0.618**	**−0.823**
	GILL	12	0.194	−0.371	**−0.678**		GILL	12	−0.192	−0.232	**0.956**
		24	**0.837**	**−0.770**	**0.994**			24	−0.268	**0.832**	**0.970**
		48	0.003	**−0.763**	−0.420			48	**0.844**	−0.279	**−0.815**
		72	**0.908**	**−0.723**	**0.994**			72	−0.467	**0.883**	**−0.867**
		96	**−0.999**	0.022	**−0.887**			96	**0.534**	**−0.558**	**−0.998**
	BRAIN	12	**0.998**	**0.847**	**0.989**		BRAIN	12	**−0.996**	0.043	**−0.559**
		24	0.095	**−0.742**	0.472			24	**0.924**	−0.364	−0.466
		48	−0.130	0.189	**0.578**			48	**−0.837**	**−0.613**	**−0.551**
		72	**−0.997**	**0.718**	−0.157			72	**0.562**	**0.769**	−0.347
		96	0.235	**0.616**	0.287			96	**−0.746**	**0.968**	**−0.999**
	MUSCLE	12	**0.965**	0.135	**−0.998**		MUSCLE	12	0.435	−0.429	0.200
		24	**0.765**	0.092	−0.267			24	**0.981**	0.391	**−0.984**
		48	**−0.822**	**0.778**	−0.165			48	−0.338	**0.512**	**−0.996**
		72	0.459	−0.464	**0.981**			72	**0.829**	**−0.928**	**0.931**
		96	**−0.717**	**0.690**	0.314			96	**−0.980**	**−0.640**	**0.661**
PCC	BLOOD	12	−0.333	**−0.996**	−0.459						
		24	**−0.951**	**0.583**	0.214						
		48	**0.811**	**0.991**	**0.896**						
		72	0.123	**0.928**	−0.248						
		96	**0.973**	−0.122	0.093						
	LIVER	12	**−0.693**	−0.354	**0.998**						
		24	−0.430	0.186	**−0.987**						
		48	**0.735**	**0.857**	−0.360						
		72	**0.800**	−0.137	**0.879**						
		96	**−0.813**	**−0.903**	**−0.979**						
	GILL	12	0.357	0.108	**0.675**						
		24	**−0.662**	**−0.999**	**0.778**						
		48	**0.769**	0.273	**−0.936**						
		72	−0.004	**−0.660**	−0.200						
		96	**−0.580**	**−0.994**	**0.925**						
	BRAIN	12	**0.781**	0.215	**0.913**						
		24	**−0.961**	**−0.982**	**−0.585**						
		48	**0.778**	**−0.746**	**−0.813**						
		72	**0.917**	**−0.732**	**−0.959**						
		96	0.184	0.160	−0.083						
	MUSCLE	12	**−0.883**	**−0.637**	**0.993**						
		24	**0.733**	**0.951**	**0.828**						
		48	**0.945**	**0.658**	**0.619**						
		72	0.217	**0.644**	**0.986**						
		96	−0.320	**−0.786**	**−0.959**						
ANTIOXIDANT ACTIVITY											
SOD	BLOOD	12	**−0.641**	**−0.787**	**−0.813**	**CAT**	BLOOD	12	−0.402	0.348	**0.772**
		24	0.215	**−0.999**	**0.989**			24	−0.102	**−0.544**	−0.263
		48	**0.992**	**0.980**	**−0.713**			48	−0.223	**0.993**	**0.992**
		72	**0.647**	**−0.514**	**0.518**			72	**0.528**	**0.821**	**−0.974**
		96	−0.496	**−0.980**	**−0.671**			96	0.348	0.433	0.032
	LIVER	12	**−0.811**	**−0.609**	**0.990**		LIVER	12	**−0.990**	**0.784**	**−0.771**
		24	**0.736**	**−0.999**	**−0.936**			24	**0.908**	**−0.770**	**−0.997**
		48	**0.756**	**−0.947**	**−0.850**			48	**−0.991**	**−0.940**	**0.566**
		72	**−0.724**	**0.854**	**0.963**			72	0.125	0.044	0.295
		96	**0.987**	**−0.962**	**−0.983**			96	**0.982**	**−1.000**	−0.089
	GILL	12	**−0.987**	**0.631**	**−0.981**		GILL	12	**0.529**	**0.834**	**−0.505**
		24	0.242	**0.947**	**0.693**			24	**0.526**	0.173	**−0.906**
		48	0.302	**−0.765**	**0.949**			48	**0.706**	**−0.998**	**0.801**
		72	**0.592**	**0.998**	0.128			72	0.496	−0.089	**0.999**
		96	**0.901**	**−1.000**	**0.794**			96	**0.690**	−0.353	**0.980**
	BRAIN	12	**−0.971**	**0.974**	**0.983**		BRAIN	12	**0.976**	**0.766**	**0.968**
		24	**0.966**	**−0.840**	**0.816**			24	**0.725**	**−0.987**	**0.731**
		48	**−0.827**	**−0.904**	**0.622**			48	**0.700**	**−0.773**	**0.915**
		72	0.109	0.447	**−0.825**			72	0.496	**−0.890**	−0.397
		96	−0.101	**−0.860**	**−0.928**			96	**−0.881**	0.011	−0.259

(*continued on next page*)

Table 2 (*continued*)

Biomarkers	Organs	Time (h)	Concentration			Biomarkers	Organs	Time (h)	Concentration		
			50 ng L^{-1}	100 ng L^{-1}	1000 ng L^{-1}				50 ng L^{-1}	100 ng L^{-1}	1000 ng L^{-1}
	MUSCLE	12	**1.000**	0.252	−0.404		MUSCLE	12	**0.888**	−0.435	**−0.646**
		24	**−0.975**	**−0.783**	**−0.897**			24	**−0.933**	**−0.812**	**0.744**
		48	−0.438	0.241	**−1.000**			48	**−0.958**	**0.613**	**−1.000**
		72	**−0.767**	0.241	**0.710**			72	**−0.526**	**−0.780**	**−0.534**
		96	**−0.907**	−0.186	−0.003			96	**−0.966**	0.246	0.497

Correlation coefficients > 0.5 are significant (shown in bold).

Table 3
Bioaccumulation factors estimated.

Test concentration	Exposure time (h)	Blood	Liver	Gill	Brain	Muscle
50 ng L^{-1} (C1)	12	0.09	0.07	0.04	0.02	0.07
	24	0.11	0.09	0.06	0.03	0.08
	48	0.13	0.10	0.08	0.05	0.10
	72	0.18	0.13	0.10	0.06	0.13
	96	0.25	0.19	0.16	0.11	0.19
100 ng L^{-1} (C2)	12	0.07	0.05	0.05	0.03	0.06
	24	0.08	0.06	0.06	0.05	0.07
	48	0.09	0.07	0.07	0.06	0.07
	72	0.13	0.10	0.10	0.08	0.10
	96	0.14	0.12	0.12	tr	0.12
1000 ng L^{-1} (C3)	12	0.06	0.04	0.03	0.02	0.04
	24	0.09	0.06	0.05	0.04	0.05
	48	0.11	0.07	0.07	0.05	0.07
	72	0.14	0.11	0.10	0.07	0.09
	96	0.18	0.13	0.12	0.10	0.12

The results of biomarkers of oxidative damage show increases in HPC and TBARS (Figs. 1 and 2) in all organs evaluated at all three test concentrations which are in the main significant. HPC increases in blood, gill and muscle were concentration dependent, while TBARS had a tendency to increase significantly at the highest (C3) concentration. The greatest increase in both biomarkers occurred in brain. Diverse factors are known to favor a high susceptibility of the central nervous system to oxidative stress: (1) brain tissue undergoes high oxidative phosphorylation and uses large amounts of oxygen; (2) it is also rich in polyunsaturated fatty acids that can be a substrate of lipid peroxidation; and (3) certain brain regions contain high concentrations of free iron which facilitate free radical formation (Olanow, 1993). Our results are similar to the findings of Tüzün et al. (1999), who reported significant increases in MDA levels in brain, liver and kidney of male albino rat neonates. HPC and MDA increases may be explained by the mechanism of action of GLB: in binding to its biological receptor in the cell membrane, this sulfonylurea elicits closing of Na+/K + -pump potassium channels which reduces the efflux of postassium, inducing cell membrane depolarization, inhibition of ATP synthesis and increased intracellular calcium (Eliasson et al., 1996). This has been associated with higher levels of extracellular glutamate (Ferrand-Drake et al., 1999) and activation of a great variety of calcium-dependent enzymes, the most important of these being proteases, lipases and endonucleases which damage the cytoskeleton, cell membrane, and nucleic acids respectively in the brain (Orrenius et al., 1989). Similarly, increased intracellular calcium has been correlated with free radical formation through activation of phospholipase A2 and nitric oxide synthase (NOS) (Lafon-Cazal et al., 1993). Increases in HPC and MDA can also be explained by the phase I biotransformation of GLB mediated by the CYP450 system which can generate free radicals such as hydroxyl radicals, singlet oxygen and superoxide. These ROS are highly reactive and can directly attack membrane lipids. The subfamily CYP2 is especially responsible for GLB biotransformation, in particular CYP2C9 (Blanco et al., 2005; Zanger et al., 2008). Diverse CYP gene families have been characterized in fish such as *C. carpio*, among others, CYP1, CYP2, CYP3, CYP4, CYP11, CYP17 and CYP19 (Stegeman and Livingstone, 1998; Botello et al., 2005). This suggests that the phase I biotransformation of GLB to 3- and 4-hydroxy-GLB may be similar to the process in mammals, yielding ROS that induce increases in these biomarkers in the organs evaluated. The gills are the first fish organs that enter in direct contact with the environment; they are also the site of acid-base regulation and the exchange of ions such as oxygen, and are able to biotransform xenobiotics (Monteiro et al., 2005). Similarly, the liver is the primary organ responsible for the biotransformation of xenobiotics such as GLB (Groop et al., 1991). Thus, since oxidative biotransformation of GLB occurs in these organs, this may explain the ROS increases and subsequent HPC and LPX increases recorded in gill and liver in our study.

Antioxidant enzyme results show increases in SOD activity (Fig. 4) at all test concentrations and exposure times in blood, liver and muscle. The same behavior was observed in gill and brain up to 72 h. As regards CAT activity (Fig. 5), increases were found at all concentrations and exposure times in all organs evaluated, but particularly in brain where this effect was concentration dependent. These results are consistent with those of Elmali et al. (2004) and Tüzün et al. (1999), who found increased SOD and CAT activity due to GLB exposure in male albino rat and male albino rat neonates respectively. The antioxidant defense system is mediated by SOD and CAT which act by scavenging ROS and converting them into less reactive and less toxic compounds. SOD is the first enzyme involved in this process and the one responsible for dismutation of the superoxide anion radical to form hydrogen peroxide (van der Oost et al., 2003). Subsequently, CAT acts on this substrate, yielding H_2O and O_2 (Vendemiale et al., 1999). Increases in SOD and CAT in the present study can be explained by the biotransformation of GLB and the mechanism of action of this compound which, as stated previously, induce formation of free radicals such as superoxide and H_2O_2 that are substrates of SOD and CAT respectively. On the other hand, the results found are similar to other pharmaceuticals previously reported, such as diclofenac (Islas-Flores et al., 2013) and ibuprofen (Islas-Flores et al., 2017).

GLB concentrations in the organs evaluated at the various exposure times indicate that the matrices in which GLB uptake was highest were blood, liver and muscle, while the organs of lowest uptake were gill and brain. The physicochemical properties of GLB make this compound poorly soluble in water (approximately 38 μmol/L at 37 °C) and with low oral bioavailability (Censi et al., 2016), which is confirmed in Tables 1 and 3 by its low bioconcentration values in the different organs. However, these low levels of GLB uptake induced oxidative stress at the various exposure times in a statistically significant manner. In the other hand, all biomarkers used were found to be correlated with the various test concentrations, suggesting the presence of oxidative stress in the organs evaluated (Table 2).

To summarize and based on our findings, GLB is suggested to induce oxidative stress in *C. carpio* through the following mechanism (Fig. 6): GLB binds to its biological receptor in the cell

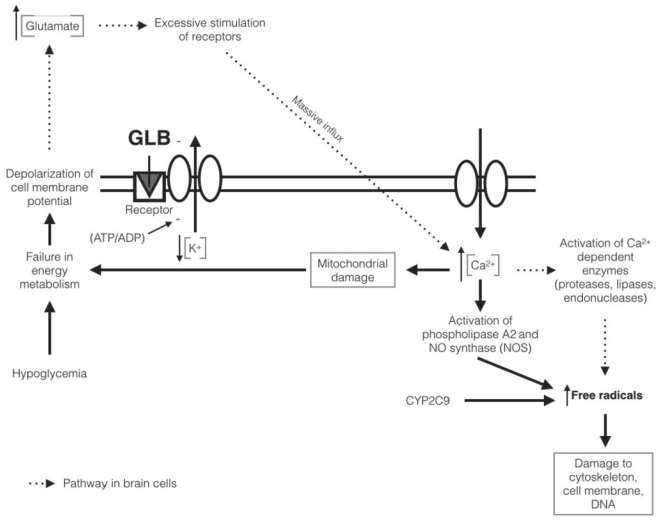

Fig. 6. Proposed mechanism of oxidative stress induction in *Cyprinus carpio*.

membrane. As a consequence, closing of Na+/K + -pump-dependent potassium channels takes place and potassium efflux is reduced, leading to cell membrane depolarization. This depolarization may lead, on the one hand, to increased intracellular calcium which activates phospholipase A2 and NOS to form free radicals, and on the other may induce, in the brain especifically, the release of neurotransmitters such as glutamate which, when accumulated, also favors increased intracellular calcium, activating calcium-dependent enzymes such as proteases, lipases and endonucleases that can in turn release free radicals. A further way in which free radicals are released is through the phase I biotransformation of GLB mediated by the CYP450 system which can originate free radical formation. Finally, these free radicals can damage the cytoskeleton, cell membrane and DNA. Oxidative stress was induced from the lowest concentrations (50 and 100 ng L^{-1}) which are environmentally relevant, so the presence of GLB in water bodies could represent a risk for the species that are in contact with this.

5. Conclusions

C. carpio bioconcentrates GLB from 12 h of exposure onward, and the organs in which this compound bioconcentrates most are blood, liver and muscle. GLB induced increases in HPC, LPX and PCC as well as SOD and CAT antioxidant activity in all organs evaluated. While the brain was one of the organs with lower GLB bioconcentration, significant increases were nevertheless observed in biomarkers of oxidative damage. The biomarkers of exposure and effect employed in the study are reliable for use in evaluating the impact of the sulfonylurea GLB in fish of commercial interest such as *C. carpio*.

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