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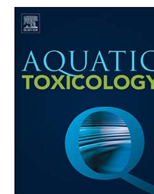

Metoprolol induces oxidative damage in common carp (*Cyprinus carpio*)

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ABSTRACT

During the last decade, β-blockers such as metoprolol (MTP) have been frequently detected in surface water, aquatic systems and municipal water at concentrations of ng/L to μg/L. Only a small number of studies exist on the toxic effects induced by this group of pharmaceuticals on aquatic organisms. Therefore, the present study aimed to evaluate the oxidative damage induced by MTP in the common carp *Cyprinus carpio*, using oxidative stress biomarkers. To this end, indicators of cellular oxidation such as hydroperoxide content (HPC), lipid peroxidation (LPX) and protein carbonyl content (PCC) were determined, as well as the activity of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT). Also, concentrations of MTP and its metabolite O-desmethyl metoprolol were determined in water as well as carp gill, liver, kidney, brain and blood, along with the partial uptake pattern of these compounds. Results show that carp takes up MTP and its metabolite in the different organs evaluated, particularly liver and gill. The oxidative stress biomarkers, HPC, LPX, and PCC, as well as SOD and CAT activity all increased significantly at most exposure times in all organs evaluated. Results indicate that MTP and its metabolite induce oxidative stress on the teleost *C. carpio* and that the presence of these compounds may constitute a risk in water bodies for aquatic species.

1. Introduction

Emerging contaminants are widely detected and are known for their potential to cause environmental impact and induce adverse effects on hydrobiont health (Barceló and López de Alda, 2008). Among the types of emerging contaminants that demand greater attention – due to lack of environmental data and ecotoxicological analytical methods as well as the possible consequences of their presence in the environment – are pharmaceuticals (Petrovic et al., 2002). The worldwide consumption of pharmaceuticals is estimated at 100,000–200,000 annual tons (Tijani et al., 2016). The most frequently prescribed agents include analgesics/anti-inflammatories such as ibuprofen, antibiotics such as amoxicillin, and antihypertensives like metoprolol (MTP) (Hernando et al., 2006).

Antihypertensives are a broad group of products among which calcium channel blockers, angiotensin-converting enzyme inhibitors, and β-blockers are prominent (He and Whelton, 1997). Within the group of β-blockers is MTP, a β-1 selective (cardioselective) adrenergic blocker (Fang et al., 2004) that competes with adrenergic neurotransmitters such as catecholamines for binding to β-adrenergic receptors. It decreases catecholamine effects on cardiac muscle, inducing cardiac frequency and contractility reduction, and as a result decreases cardiac output, arterial blood pressure and myocardial oxygen consumption. MTP is prescribed to treat hypertension (high blood pressure), relieve angina (chest pain) and prevent heart attacks (Fang et al., 2004).

In the last decade β-blockers such as MTP have been frequently detected in aquatic systems, surface water and municipal water at concentrations of ng/L to μg/L (Kümmerer, 2010). In Germany 1.2 μg/L have been detected in wastewater treatment plant effluent (Bernhard et al., 2017), France reports concentrations of 15.8–435 ng/L in the effluent of 14 wastewater treatment plants (Gabet-Giraud et al., 2010). In Brazil, levels of up to 9.9 μg/L were detected in hospital effluents (Wilde et al., 2012). In addition, concentrations of up to 0.35 μg/L have been reported in groundwater (López-Serna et al., 2013) and up to 0.038 μg/L in drinking water (López-Serna et al., 2010) in Spain. Finally, Mexico reports concentrations of 200–290 ng/L in the Mezquital

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Valley (Siemens et al., 2008).

Despite reports of MTP concentrations in aquatic ecosystems throughout the world, few studies have examined the toxic effects of this compound on organisms present in water bodies, probably because it has not been described as an important toxic agent in the literature. Several studies have demonstrated that environmental exposures to pharmaceuticals may result in accumulation of the compounds, their metabolites, or both in tissues of aquatic organisms. Moreno-González et al. (2016) reported the bioaccumulation of β-blockers (atenolol, carazolol, metoprolol, nadolol, propranolol, sotalol) in muscle (20.3–53.6 ng/g dry weight) and liver (44.2–192.2 ng/g dry weight) of golden grey mullet (*Liza aurata*) that was collected of the Mar Menor lagoon in the Mediterranean Sea (SE Spain). The quantification of pharmaceuticals in tissues is important to know their distribution within the organism (Ramirez et al., 2007).

Van den Brandhof and Mortforts (2010) reported an LC_{50} of 150.0 mg/L for MTP in embryos of *Danio rerio* after 72 h of exposure, while a study by Sun et al. (2014), using the same bioindicator, found a 96-h LC_{50} of 167 mg/L. In both studies, reduced heart rate and increased embryo mortality occurred. A third study found an LC_{50} of 130 mg/L in rainbow trout (*Onchorhynchus mykiss*) and a NOEC (no observed effect concentration) of 32 mg/L (Moermond, 2014). On the other hand, MTP toxicity has been evaluated not only in fish, but also in algae, specifically the green alga *Pseudokirchneriella subcapitata* in which a LOEC (lowest observed effect concentration) of 7.5 mg/L was found at 72 h; both evaluations were conducted according to the guidelines proposed in OECD 201 (Moermond, 2014).

MTP undergoes biotransformation by the activity of the microsomal cytochrome P450 (CYP) monooxygenase system and specifically by the CYP2D6 subfamily (Cozza and Armstrong, 2001). Production and release of free radicals has been reported during MTP metabolism, particularly of reactive oxygen species (ROS) such as the superoxide anion radical (O_2^-) based on formation of the ternary oxy-cytochrome complex and hydrogen peroxide (H_2O_2) via the peroxy-cytochrome complex (Halpert et al., 1986). Oxidative stress is an imbalance between ROS production and antioxidant defenses that leads to a variety of physiological and biochemical changes in carbohydrates, lipids, proteins and nucleic acids, ultimately inducing cell degeneration and death (Lushchak, 2011; Regoli and Giuliani, 2014).

The common carp *Cyprinus carpio* is one of the first aquatic species in the world cultured for human consumption, and Mexico is among the top countries producing and commercializing this fish. According to the United Nations Food and Agricultural Organization, this is the most frequently cultured species in the world and the one most consumed by the human population (FAO, 2009). *C. carpio* is an excellent bioindicator of water quality due to its easy reproduction and maintenance in the laboratory, sensitivity to diverse xenobiotics and tolerance of high temperatures, as well as its economic and nutritional importance and wide geographic distribution (De la Lanza et al., 2000; Ondarza et al., 2010).

The present study aimed to evaluate the toxicity induced by three concentrations of MTP (10 ng/L, 10 µg/L and 10 mg/L, the first two have been detected environmentally, the third has been associated with diverse toxic effects in aquatic species) in brain, gill, liver, kidney and blood of the freshwater teleost *C. carpio*, using oxidative stress biomarkers.

2. Materials and methods

2.1. Reagents

Unless otherwise stated, reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Metoprolol tartrate (MTP) (CAS 56392-17-7, > 98% purity) $C_{34}H_{56}N_2O_{12}$, 684.81 MW and O-desmethyl metoprolol (O-DMM) (CAS 62572-94-5, > 98% purity) $C_{14}H_{23}NO_3$, 253.34 MW. Each of these compounds was dissolved in deionized water to prepare stock solutions from which all test concentrations were obtained.

Deionized water was used in all experiments. Sodium tetraborate decahydrate was purchased from Caledon Laboratory Chemicals (Caledon, ON, Canada); sodium hydroxide, hydrochloric acid and HPLC-grade methanol were obtained from Fisher Scientific (Nepean, ON, Canada).

2.2. Quantification of MTP and O-DMM by capillary electrophoresis (CE)

Specific determination of MTP and O-DMM in tissue and water samples was made using a P/ACE™ MDQ CE system (Beckman Coulter, Brea, CA) equipped with UV and photodiode array (PDA) detectors. Anion separations were carried out using a fused-silica capillary (50 µm internal diameter, 60 cm total length, 50 cm effective length) coated with polybrene (Polymicro Technologies, Phoenix, AZ). Samples were injected hydrodynamically for 10 s at 1.0 psi. The injection volume was set to 50 µL. All experiments were performed at 25 °C, and in triplicate.

Throughout the experiment, the capillary was rinsed sequentially with 0.1 M NaOH for 4 min, 18 MΩ water for 1 min, and run buffer for 5 min before each run. Separation conditions were maintained for 11 min until the baseline remained constant.

The inlet end of the capillary was held at a positive potential and the outlet end at a negative potential. The distance between working electrode and capillary outlet was set to 150 µm with the help of an optical microscope. The detection was set at a wavelength of 214 nm.

The software 32 Karat v8.0 was used to control the system during all determinations as well as for data treatment.

2.3. Preparation of samples

For each exposure time (12, 24, 48, 72 and 96 h), 0.5 g of each organ (brain, gill, liver and kidney) was weighed and 0.5 mL of blood was measured, as well as 50 µL water from the three test concentrations used (10 ng/L, 10 µg/L and 10 mg/L; hereafter called C1, C2 and C3 respectively).

The homogenization-deproteinization method used for MTP and O-DMM extraction followed the procedure proposed by Brooks (1983). Fresh organ samples were homogenized and mixed with phosphate buffer solution (PBS), frozen at −20 °C and protected from natural light until determination. The frozen samples were brought to ambient temperature and supplemented with 150 µL Milli-Q water and 50 µL of 70% perchloric acid. All solutions were passed through 0.45-µm Nylon membrane filters (Canadian Life Science, Peterborough, ON). MTP and O-DMM determination was carried out by CE analysis.

2.4. Preparation of standard solutions

Standard solutions of MTP and O-DMM were prepared using appropriate quantities of each compound. The compounds were dissolved in 18 MΩ water to obtain a final concentration of 10 mg/mL. Stock solutions of both compounds were stored at 4 °C and brought to ambient temperature prior to use.

2.5. Method optimization

Since MTP and O-DMM are similar in structural and physicochemical properties, the optimum conditions for their detection by CE were: sodium tetraborate decahydrate pH 11.0 at a concentration of 40 mM as the run buffer, optimum separation voltage 15 kV for 11 min run under normal polarity.

2.6. Determination of partial uptake pattern (UP)

Partial UP of MTP and O-DMM were determined at the different exposure times in all organs evaluated, using the following equation:

UP = MTP or O-DMM concentration in the organ evaluated/MTP or O-DMM concentration in water

2.7. Specimen maintenance and culture

Three-month-old juveniles of common carp (*Cyprinus carpio*), 18.4 ± 0.31 cm long and weighing 50.7 ± 7.8 g, were obtained from the aquaculture center in Tiacaque (State of Mexico) and transported to the laboratory in fully sealed polyethylene bags with enough oxygen to ensure survival. Fifteen carps were placed in 120-L tanks containing dechlorinated tap water previously reconstituted with salts: NaHCO3 (174 mg/L; Sigma-Aldrich, Toluca, Mexico), MgSO4 (120 mg/L; Sigma-Aldrich, St. Louis, Missouri, USA), KCl (8 mg/L; Vetec-Sigma-Aldrich, St. Louis, Missouri, USA), and CaSO4.2H2O (120 mg/L; Sigma- Aldrich, Toluca, Mexico) and were acclimated to test conditions for 30 days prior to the experiment.

During acclimation, carp were fed Pedregal Silver™ fish food, and three-quarters of the tank water were replaced every 24 h to maintain a healthy living environment. The physicochemical characteristics of dechlorinated tap water reconstituted with salts were maintained at a temperature of 20 ± 2 °C, oxygen concentration 80–90% of air saturation, pH 7.5-8.0, total alkalinity 17.8 ± 7.3 mg/L and total hardness 18.7 ± 0.6 mg/L. A natural light/dark (12:12 h) photoperiod was maintained. During acclimation, fish were not exposed to any pharmaceutical product or other contaminant.

2.8. Evaluation of sublethal toxicity for subsequent oxidative stress assessment

Sublethal toxicity assays involved adding MTP to conditioned water (tap water and salts) using environmentally relevant concentrations (10 ng/L, 10 μg/L) and a high concentration (10 mg/L) that has been associated with diverse toxic effects in aquatic species, in three test systems set up in 60-L plastic containers, with six juvenile carps each. A kinetics separately was run for the following exposure times: 12, 24, 48, 72 and 96 h. Exposures were static, i.e. no food was provided to specimens and no changes of water were made during the experiment. An MTP-free control system with six carps was set up for each exposure time, and all assays were performed in triplicate, using a total of 360 fish.

At the end of each exposure time, carps were removed from the systems and placed in a fish tank containing 50 mg/L clove oil as an anesthetic (Yamanaka et al., 2011) in tap water reconstituted with salts previously indicated in the Section 2.7. Anesthetized specimens were placed in lateral position and blood was obtained with a heparinized 1-mL hypodermic syringe by puncturing the caudal vessel laterally near the base of the caudal peduncle, at mid-height of the anal fin and ventral to the lateral line. Blood samples were collected in heparinized tubes, placed in phosphate buffer (pH 7.4) and sonicated, then stored at −70 °C prior to being analyzed.

After puncture, specimens were euthanized and placed in an ice bath. The gill, brain, liver and kidney were removed and placed in phosphate buffer (0.138 M NaCl, 0.0027 M KCl) at pH 7.4, the organs were homogenized separately, and then centrifuged at 12,500*g* and 4 °C for 15 min. Tissue samples were stored at −70 °C prior to being analyzed.

To evaluate cellular oxidation, the following biomarkers were used: hydroperoxide content (HPC), lipid peroxidation (LPX) and protein carbonyl content (PCC), while antioxidant defense was evaluated by measuring the activity of the enzymes superoxide dismutase (SOD) and catalase (CAT). All bioassays were performed on the supernatant.

2.9. Evaluation of cellular oxidation indicators

2.9.1. Determination of HPC

HPC was determined at 560 nm using the Jiang et al. (1992) method. Supernatant (100 μL) previously deproteinized with trichloroacetic acid (TCA) was supplemented with 900 μL of a reaction mixture consisting in 0.25 mM $FeSO_4$, 25 mM H_2SO_4, 0.1 mM xylenol orange and 4 mM of 90% butylhydroxytoluene in methanol. The mixture was allowed to stay for 60 min at ambient temperature and light protected. Results were expressed as nM cumene hydroperoxide/mg protein.

2.9.2. Determination of LPX

The method proposed by Büege and Aust (1978) was used to determine LPX. Tris-HCL buffer solution (pH 7.4) was added to 100 μL of supernatant to attain a final volume of 1 mL. Samples were incubated at 37 °C for 30 min, supplemented with 2 mL TBA-TCA reagent [0.375% thiobarbituric acid (TBA, Fluka, Sigma-Aldrich, Toluca, Mexico) in 15% TCA] and homogenized. Next, they were heated to boiling for 45 min in a double-boiler, allowed to cool and the precipitate removed by centrifuging at 3000xg for 10 min. Absorbance was read at 535 nm against a reaction blank. Malondialdehyde (MDA) content was estimated using the MEC (molar extinction coefficient) of MDA (1.56×10^5/M/cm). Results were expressed as mM MDA/mg protein.

2.9.3. Determination of PCC

2,4-Dinitrophenylhydrazine (150 μL; 10 mM DNPH in 2 M HCl) was mixed with 100 μL of supernatant. The mixture was incubated for 60 min at ambient temperature and light protected. Next, 500 μL of 20% TCA was added and the sample was left to rest for 15 min at 4 °C, then centrifuged at 1100 g for 5 min. The supernatant was discarded and the precipitate was washed four times with 1 mL ethanol:ethyl acetate (1:1), then dissolved in 1 mL guanidine (6 M, pH 2.3) and incubated at 37 °C for 30 min, as described in the Levine et al. (1994) method, modified by Parvez and Raisuddin (2005) and Burcham (2007). PCC was determined at 366 nm and results were estimated using the MEC of 21,000 M/cm. Data were expressed as nM of reactive carbonyls (C═O) formed/mg protein.

2.10. Evaluation of antioxidant defense indicators

2.10.1. Determination of SOD activity

SOD activity was determined by the Marklund and Marklund (1974) method, which is based on the capacity of SOD to inhibit autooxidation of pyrogallol, the oxidized form of which absorbs light at 420 nm. Samples were delipidated by adding 30 μL chloroform and 50 μL methanol to each 100-μL sample, agitating for 1 min and centrifuging for 15 min at 6000 g. The supernatant was removed and frozen until assayed. In a cuvette, 100 μL of delipidated sample, 2.8 mL Tris-HCl buffer solution pH 8.2 and 50 μL of an EDTA solution were mixed. Prior to the reading, 50 μL of a pyrogallol solution was added and, following reaction for 10 s, oxidation difference (OD)/min was determined.

2.10.2. Determination of CAT activity

CAT activity was determined at 240 nm by evaluating the decrease of absorbance of H_2O_2. Supernatant (20 μL) was mixed with 420 μL isolation buffer solution (0.3 M saccharose, 1 mM EDTA, 5 mM HEPES, 5 mM KH_2PO_4) and 0.2 mL H_2O_2 (20 mM), as described in the Radi et al. (1991) method. Results were expressed as mM H_2O_2/mg protein.

2.11. Determination of total protein content

Total protein content was determined by the Bradford (1976) method. Supernatant (25 μL) was supplemented with 75 μL water and 2.5 mL Bradford's reagent (0.05 g Coomassie Blue, 2.5 mL of 96% ethanol, 50 mL H_3PO_4, 500 mL water). Solutions were homogenized, then left for 5 min prior to reading absorbance at 595 nm and the concentrations read from bovine albumin standard curve.

Table 1
Performance characteristics of the method.

Compounds	Linear range (μg/L)	Calibration Curves		*R*	LOD (μg/L)	LOQ (μg/L) in samples
		Slope	Intercept			
MTP	0.00001–0.0002	1187	16368	0.9855	0.0001	0.0002
O-DMM	0.00001–0.0005	2944	13830	0.9972	0.0001	0.0002

LOD = limit of detection; LOQ = limit of quantification.

Statistical analysis

Oxidative stress biomarker results were evaluated by one-way analysis of variance (ANOVA), followed by a multiple comparison test (Bonferroni) with *P*-value < *0.05*. Statistical determinations were made with SPSS v10 software (SPSS, Chicago, IL). Pearson's correlation analysis was used to examine potential correlations between partial UP of MTP and O-DMM at 12, 24, 48, 72 and 96 h, and oxidative stress biomarkers in blood, brain, gill, liver and kidney of *C. carpio*.

3. Results

3.1. Quantification of MTP and O-DMM by CE

Under optimum detection conditions, MTP and O-DMM migration times were 3.62 min and 3.69 min, respectively. Calibration curves were calculated by plotting peak area values against MTP or O-DMM concentration. The performance characteristics of the method are listed in Table 1. The MTP and O-DMM regression coefficients were 0.9855 and 0.9972, respectively, considering a LOD of 0.0001 μg/L and a LOQ of 0.0001 μg/L, in both cases.

Table 2 lists the results of MTP and O-DMM determination by CE analysis in the different matrices evaluated. The highest concentrations of MTP and O-DMM in water were found at 12 h and decreased as the exposure time increased. In the organs (brain, gill, liver and kidney) and blood it can be observed that the concentration of MTP and O-DMM increases as the exposure time increases.

3.2. Partial UP in the organs evaluated

Table 3 shows the partial UP obtained for MTP and O-DMM in gill, liver, kidney, brain and blood. These values ranged between 0.08 and 3.50 in the different organs evaluated, being highest in gill and liver.

3.3. Evaluation of oxidative stress

3.3.1. HPC

HPC results are shown in Fig. 1. Significant increases with respect to the control group (*P* < *0.05*) were observed in brain at 48 and 96 h with C1; 12, 24 and 48 h with C2; and 24, 48 and 72 h with C3, in gill at 12 and 24 h with C1; 12, 24 and 48 h with C2; and 12, 48, 72 and 96 h with C3, in liver at 12, 48 and 96 h with C1; and 12, 24, 48, 72 and 96 h with C2 and C3, as well as in kidney at 24 and 96 h with C1 and C3; 12, 48 and 72 h with C2, and finally, in blood at 12, 72 and 96 h with C1; 12, 24, 48, 72 and 96 h with C2; and 96 h with C3. The highest HPC value was recorded in blood at 96 h with C3. In contrast, significant decreases relative to the control group were found in brain at 96 h with C2; and gill at 96 h with C1.

3.3.2. LPX

LPX results are shown in Fig. 2. Significant increases with respect to the control group (*P* < *0.05*) were found in brain at 12, 24, 48, 72 and 96 h with C1 and C3; at 12, 24, 48 and 96 h with C2, in gill at 24 h with C1; 48, 72 and 96 h with C2; 12, 24, 72 and 96 h with C3, in liver at 48 and 96 h with C1; 24, 72 and 96 h with C2; 12, 24, 48, 72 and 96 h with C3, in kidney at 12, 24 and 72 h with C1; 12, 24, 48 h with C2; 12, 24, 48 and 96 h with C3, as well as in blood at 12, 24, 48 and 96 h with C1; 12, 24, 48, 72 and 96 h with C2; 12, 48, 72 and 96 h with C3. The highest LPX value was recorded in blood at 72 h with C2.

3.3.3. PCC

PCC results are shown in Fig. 3. Significant increases with respect to the control group *(P* < *0.05*) were observed in brain at 24, 48 and 96 h with C1; 12, 24 and 96 h with C2; 12, 24, 48, 72 and 96 h with C3, in gill at 24 h with C1; 24, 48 and 96 h with C2; 12, 72 and 96 h with C3, in liver at 24, 48, 72 and 96 h with C1; 12, 24, 48, 72 and 96 h with C2; 24, 48, 72 and 96 h with C3, as well as in kidney at 24 h with C1; 12 h

Table 2
Determination of metoprolol (MTP) and O-desmethyl metoprolol (O-DMM) by capillary electrophoresis.

	Units		12 h		24 h		48 h		72 h		96 h	
			MTP	O-DMM	MTP	O-DMM	MTP	O-DMM	MTP	O-DMM	MTP	O-DMM
C1	(ng/L)	Water	2.60 ± 0.09	1.80 ± 0.12	2.10 ± 0.05	1.70 ± 0.11	2.00 ± 0.09	1.20 ± 0.10	1.30 ± 0.13	0.70 ± 0.07	0.50 ± 0.09	0.20 ± 0.09
	(ng/g of wet tissue)	Brain	0.40 ± 0.07	0.40 ± 0.04	0.60 ± 0.09	0.60 ± 0.08	0.60 ± 0.06	0.40 ± 0.08	0.60 ± 0.12	0.60 ± 0.09	1.00 ± 0.09	0.80 ± 0.06
		Gill	0.40 ± 0.08	0.40 ± 0.08	0.40 ± 0.08	0.40 ± 0.03	1.20 ± 0.06	0.40 ± 0.02	0.80 ± 0.12	1.00 ± 0.04	1.40 ± 0.11	1.40 ± 0.08
		Liver	0.80 ± 0.11	0.40 ± 0.05	0.80 ± 0.11	0.60 ± 0.09	0.80 ± 0.08	0.40 ± 0.07	0.80 ± 0.14	1.40 ± 0.07	1.20 ± 0.10	0.40 ± 0.10
		Kidney	0.40 ± 0.09	0.40 ± 0.07	0.40 ± 0.12	0.40 ± 0.04	0.60 ± 0.04	0.60 ± 0.04	0.40 ± 0.12	0.60 ± 0.10	0.40 ± 0.06	0.40 ± 0.10
	(ng/L)	Blood	0.30 ± 0.10	0.30 ± 0.07	0.40 ± 0.09	0.30 ± 0.06	0.30 ± 0.03	0.60 ± 0.10	0.20 ± 0.08	0.60 ± 0.04	0.50 ± 0.11	0.30 ± 0.06
C2	(μg/L)	Water	1.90 ± 0.10	1.00 ± 0.10	1.70 ± 0.09	1.00 ± 0.12	1.30 ± 0.07	0.80 ± 0.09	1.10 ± 0.12	0.30 ± 0.09	0.70 ± 0.09	0.30 ± 0.10
	(μg/g of wet tissue)	Brain	0.40 ± 0.08	0.40 ± 0.03	0.80 ± 0.09	0.40 ± 0.06	0.80 ± 0.05	0.60 ± 0.09	0.80 ± 0.10	0.80 ± 0.05	1.60 ± 0.12	0.40 ± 0.09
		Gill	0.60 ± 0.09	0.40 ± 0.06	0.60 ± 0.06	0.60 ± 0.08	1.40 ± 0.07	1.40 ± 0.08	1.00 ± 0.09	1.00 ± 0.10	1.00 ± 0.14	1.00 ± 0.05
		Liver	0.40 ± 0.07	0.40 ± 0.06	0.80 ± 0.08	0.40 ± 0.05	1.20 ± 0.10	1.20 ± 0.06	1.00 ± 0.11	0.80 ± 0.05	0.80 ± 0.13	0.40 ± 0.09
		Kidney	0.40 ± 0.11	0.40 ± 0.07	0.40 ± 0.06	0.40 ± 0.08	0.40 ± 0.11	0.80 ± 0.09	0.40 ± 0.03	0.60 ± 0.06	1.00 ± 0.12	1.20 ± 0.07
	(μg/L)	Blood	0.20 ± 0.06	0.20 ± 0.07	0.20 ± 0.04	0.20 ± 0.04	0.40 ± 0.13	0.20 ± 0.05	0.20 ± 0.11	0.20 ± 0.10	0.30 ± 0.08	0.20 ± 0.09
C3	(mg/L)	Water	2.60 ± 0.12	1.30 ± 0.10	2.40 ± 0.16	1.20 ± 0.09	2.10 ± 0.09	0.80 ± 0.07	1.10 ± 0.10	0.40 ± 0.08	0.70 ± 0.13	0.30 ± 0.10
	(mg/g of wet tissue)	Brain	0.40 ± 0.07	0.40 ± 0.07	0.40 ± 0.08	0.40 ± 0.08	0.80 ± 0.04	0.60 ± 0.06	1.00 ± 0.11	0.40 ± 0.09	1.00 ± 0.09	0.80 ± 0.08
		Gill	0.60 ± 0.07	0.40 ± 0.04	1.00 ± 0.07	0.60 ± 0.08	0.60 ± 0.11	1.20 ± 0.09	1.20 ± 0.09	0.80 ± 0.10	1.20 ± 0.11	1.00 ± 0.09
		Liver	0.60 ± 0.09	0.40 ± 0.06	1.20 ± 0.10	0.40 ± 0.05	0.40 ± 0.09	0.20 ± 0.07	0.60 ± 0.10	1.00 ± 0.09	1.40 ± 0.09	0.60 ± 0.10
		Kidney	0.40 ± 0.10	0.40 ± 0.06	0.60 ± 0.07	0.40 ± 0.03	0.60 ± 0.05	0.60 ± 0.06	0.80 ± 0.11	0.80 ± 0.05	0.80 ± 0.10	1.00 ± 0.10
	(mg/L)	Blood	0.20 ± 0.09	0.20 ± 0.05	0.30 ± 0.06	0.20 ± 0.03	0.60 ± 0.07	0.20 ± 0.05	0.40 ± 0.09	0.20 ± 0.06	0.30 ± 0.06	0.20 ± 0.06

Table 3
Partial uptake patterns of metoprolol (MTP) and its metabolite O-desmethyl metoprolol (O-DMM).

		12 h		24 h		48 h		72 h		96 h	
		MTP	O-DMM	MTP	O-DMM	MTP	O-DMM	MTP	O-DMM	MTP	O-DMM
C1	**Brain**	0.08	0.11	0.14	0.18	0.15	0.17	0.23	0.43	1.00	2.00
	Gill	0.08	0.11	0.24	0.12	0.30	0.42	0.31	0.71	1.40	3.50
	Liver	0.15	0.11	0.19	0.18	0.20	0.42	0.31	1.00	1.20	3.50
	Kidney	0.08	0.11	0.10	0.12	0.15	0.25	0.15	0.43	0.40	1.00
	Blood	0.12	0.17	0.19	0.18	0.15	0.50	0.15	0.86	1.00	1.50
C2	**Brain**	0.11	0.20	0.24	0.20	0.31	0.38	0.36	1.33	1.14	0.67
	Gill	0.16	0.20	0.18	0.30	0.54	0.88	0.45	1.67	1.43	1.67
	Liver	0.11	0.20	0.24	0.20	0.46	0.75	0.45	1.33	1.29	2.33
	Kidney	0.11	0.20	0.12	0.20	0.15	0.50	0.18	1.00	0.71	2.00
	Blood	0.11	0.20	0.12	0.20	0.31	0.25	0.18	0.67	0.43	0.67
C3	**Brain**	0.08	0.15	0.08	0.17	0.19	0.38	0.45	0.50	0.71	1.33
	Gill	0.12	0.15	0.21	0.25	0.38	0.75	0.55	1.00	0.86	1.67
	Liver	0.12	0.15	0.25	0.17	0.33	0.75	0.73	1.25	1.00	2.67
	Kidney	0.08	0.15	0.13	0.17	0.14	0.38	0.36	1.00	0.57	1.67
	Blood	0.08	0.15	0.13	0.17	0.29	0.25	0.36	0.50	0.43	0.67

with C2; 24 and 96 h with C3, and finally, in blood at 12, 24, 48 and 96 h with C1; 24, 48, 72 and 96 h with C2; 12, 24, 48, 72 and 96 h with C3. The highest PCC value was recorded in blood at 48 h with C2. In contrast, a significant decrease with respect to the control group was found in kidney at 48 h with C3.

3.4. Evaluation of antioxidant enzyme activities

3.4.1. SOD activity

SOD activity results are shown in Fig. 4. Significant increases with respect to the control group ($P < 0.05$) were found in brain at 12, 24 and 72 h with C1; 12, 48, 72 and 96 h with C2; 12, 24, 48, 72 and 96 h with C3, in gill at 12, 24, 48 h with C1 and C2; 12, 24 and 72 h with C3, in liver at 12, 24, 48 and 96 h with C1; 12, 48, 72 and 96 h with C2; 12, 48 and 96 h with C3, in kidney at 12, 48 and 96 h with C1; 12, 72 and 96 h with C2; 12, 24, 48, 72 and 96 h with C3, as well as in blood at 12, 24 and 48 h with C1; 12, 48, 72 and 96 h with C2; 12, 24, 48, 72 and 96 h with C3. The highest SOD activity value was recorded in blood at 12 h with C2.

3.4.2. CAT activity

CAT activity results are shown in Fig. 5. Significant increases with respect to the control group ($P < 0.05$) were observed in brain at 96 h with C1; 24, 48, 72 and 96 h with C2; 24, 72 and 96 h with C3, in gill at 12 and 24 h with C1; 48 and 72 h with C2; 12, 24, 72 and 96 h with C3, in liver at 12, 24, 48 and 72 h with C1; 24, 48, 72 and 96 h with C2; 12, 24, 48, 72 and 96 h with C3, as well as in kidney at 24, 48 and 72 h with C1; 12, 24, 48, 72 and 96 h with C2; 12, 24, 48 and 96 h with C3, and finally, in blood at 24, 48 and 96 h with C1; 24 and 96 h with C2; 12, 24, 48, 72 and 96 h with C3. The highest CAT activity was recorded in blood at 12 h with C3. In contrast, significant decreases with respect to the control group were observed in liver at 12 h with C2 and kidney at 96 h with C1.

The percentages of increases and decreases of oxidative damage and antioxidant activity are shown in Table 4.

4. Discussion

A large number of analytical methods have been developed to identify and quantify diverse β-blockers in aquatic systems (Andreozzi et al., 2003). Nevertheless, data of environmental occurrence for these compounds are scarce. Possible reasons for this are the lack of very sensitive analytical methods and the fact that some β-blockers are transformed by both abiotic and biotic factors, yielding metabolites and degradation products that can be harder to detect than the parent compound, and whose toxicity has not yet been defined.

In the present study, MTP degraded rapidly to O-DMM at all three concentrations from 12 h onward. Such degradation may have been due to biotic characteristics of the system (biotransformation of MTP by the CYP in carp) or abiotic characteristics (MTP photodegradation).

Lubes (2009) used molecular modeling to explain MTP biotransformation by means of the semi-empirical parametric method 3, considering electrostatic potential maps, HOMO/LUMO (highest occupied/lowest occupied molecular orbital) energies, dipole moments, heats of formation, and partition coefficient. He concluded that the three main metabolites of MTP are O-DMM, N-dealkyl metoprolol and α-hydroxy metoprolol (α-OHM). Lubes also concluded that the enthalpies of formation of MTP metabolites are more negative than MTP itself, suggesting that this pharmaceutical can be readily degraded to any of its metabolites since they are thermodynamically more stable. Belpaire et al. (1998) had stated previously that biotransformation of MTP in human microsomes is carried out by CYP2D6, a CYP isoenzyme. Similarly, Hoffmann et al. (1980) reported that the CYP2D6 subfamily is responsible for the biotransformation of approximately 70% of MTP and that the main biotransformation metabolites are O-DMM (65%), α-OHM (10%) and N-dealkyl metoprolol (< 10%). Stegeman and Livingstone (1998) identified families of CYP genes in diverse fish species including *C. carpio*. The subfamilies found by Stegeman and Livingstone include CYP1, CYP2, CYP3, CYP4, CYP11, CYP17 and CYP19, and specifically the CYP2D6 subfamily. Results obtained in our study confirm that MTP is biotransformed into O-DMM, suggesting that the route of transformation in carp is similar to the route in humans.

Furthermore, Triebskorn et al. (2007) found that MTP causes ultrastructural effects such as collapse of cellular compartmentalization and glycogen reduction in liver of rainbow trout exposed to 1 μg/L. The authors concluded that these structural reactions may indicate activation of enzymes equivalent to mammalian CYPs and therefore induction of biotransformation in the fish liver.

On the other hand, certain abiotic factors such as photolysis and UV radiation can affect the environmental fate of MTP (Liu and Williams, 2007; Piram et al., 2008). These authors showed that MTP is degraded in the presence of natural light from 0.0011 h onward with half-life time of 630 h. In the case of photolysis, the main degradation products identified are O-DMM and α-OHM (Borkar et al., 2012).

Both biotransformation and photolysis degradation studies support our results, which indicate that MTP is rapidly transformed (12 h) into O-DMM, as shown in Table 2.

In our study, MTP and O-DMM concentrations in water decreased as exposure time increased, while increase of tissue levels of both compounds were found from 12 h on. These data indicate that MTP and its metabolite were taken up in the tissues assayed. The accumulation of xenobiotics varies among aquatic species depending on the exposure

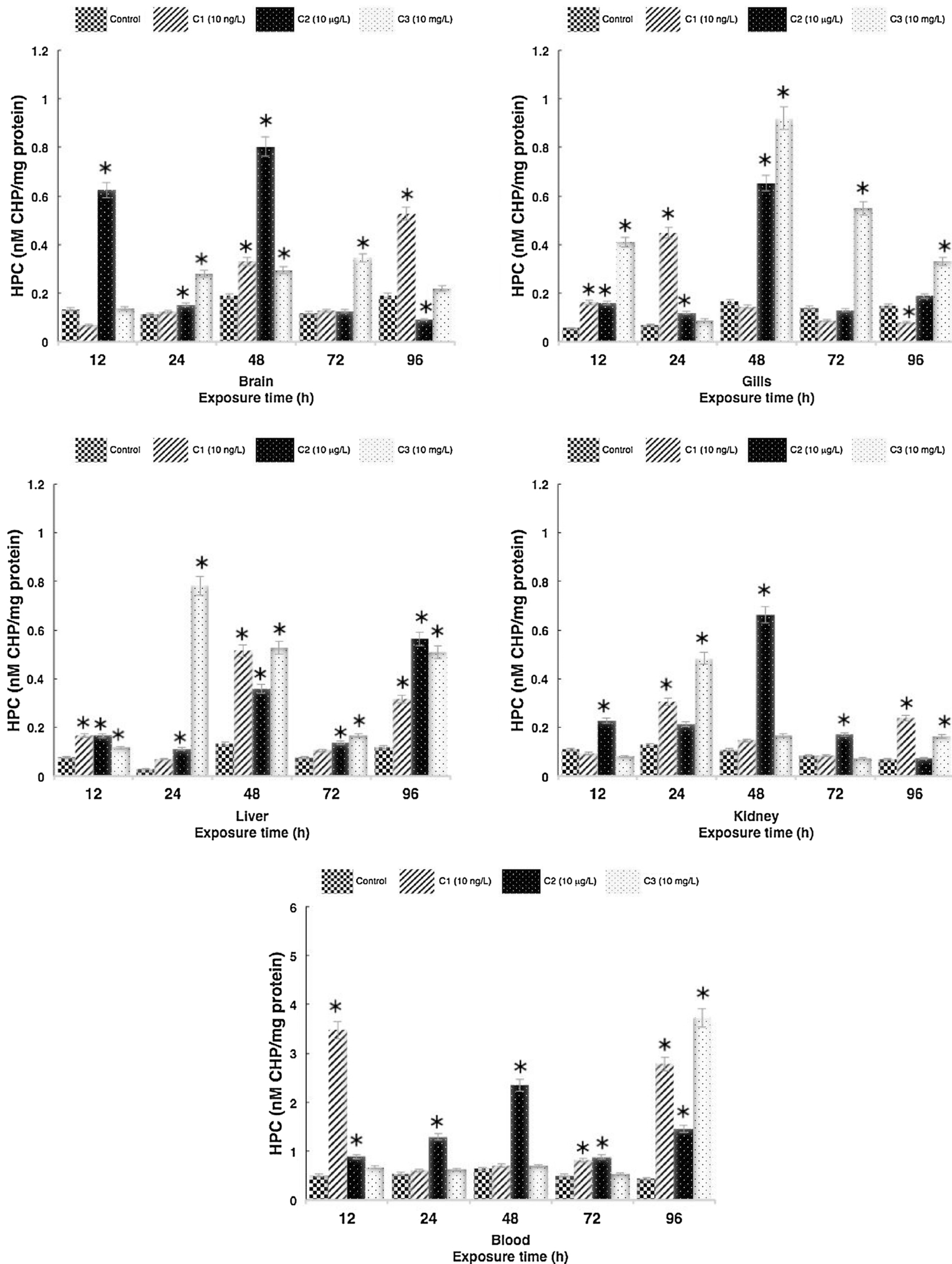

Fig. 1. Hydroperoxide content (HPC) in brain, gill, liver, kidney and blood of *Cyprinus carpio* exposed to three different concentrations of metoprolol (C1, C2 and C3) for 12, 24, 48, 72 and 96 h. Values are the mean of three replicates ± SE. CHP = cumene hydroperoxide. *Significant differences with the control group, ANOVA and Bonferroni ($P < 0.05$).

conditions of test systems. Our results are consistent with those of Contardo-Jara et al. (2010), who found that MTP has a high bioconcentration factor (BCF = 0.534) in mussel *Dreissena polymorpha* exposed to different concentrations of this pharmaceutical in flow-through systems for 4 and 7 days. The BCFs of pharmaceuticals are affected by xenobiotic liposolubility and the biological half-life (metabolism/excretion) of the compound in the body (Brown et al., 2007). MTP has a partition coefficient (log Kow) of 1.88 (Hansch et al., 1995), which indicates that it is moderately lipophilic and has a high capacity of accumulation in tissues in protonated form, as shown in Table 3.

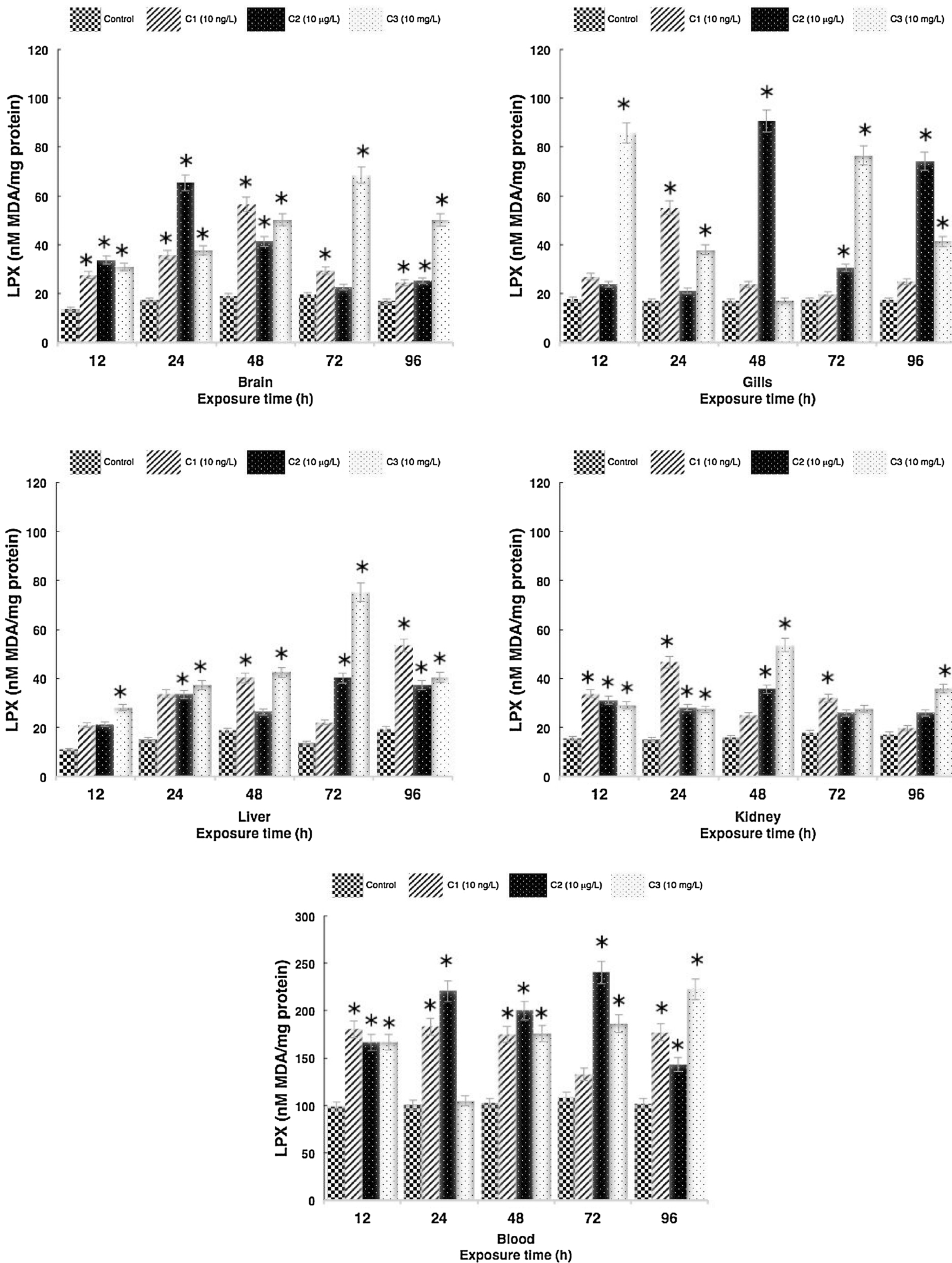

Fig. 2. Lipid peroxidation (LPX) in brain, gill, liver, kidney and blood of *Cyprinus carpio* exposed to three different concentrations of metoprolol (C1, C2 and C3) for 12, 24, 48, 72 and 96 h. Values are the mean of three replicates ± SE. MDA = malondialdehyde. *Significant differences with the control group, ANOVA and Bonferroni ($P < 0.05$).

Both MTP and O-DMM were accumulated in the organs evaluated, particularly liver and gill from 12 h onward. Since these organs have high metabolic activity, it is reasonable to find that these compounds were accumulated preferably in the liver and gill in comparison to other tissues assayed in our study.

The increases found in MTP and O-DMM concentrations at the different exposure times in the organs evaluated concur with the increases observed in indicators of cellular oxidation and antioxidant defense in *C. carpio*, starting at 12 h of exposure.

The CYP-mediated biotransformation of MTP is directly associated

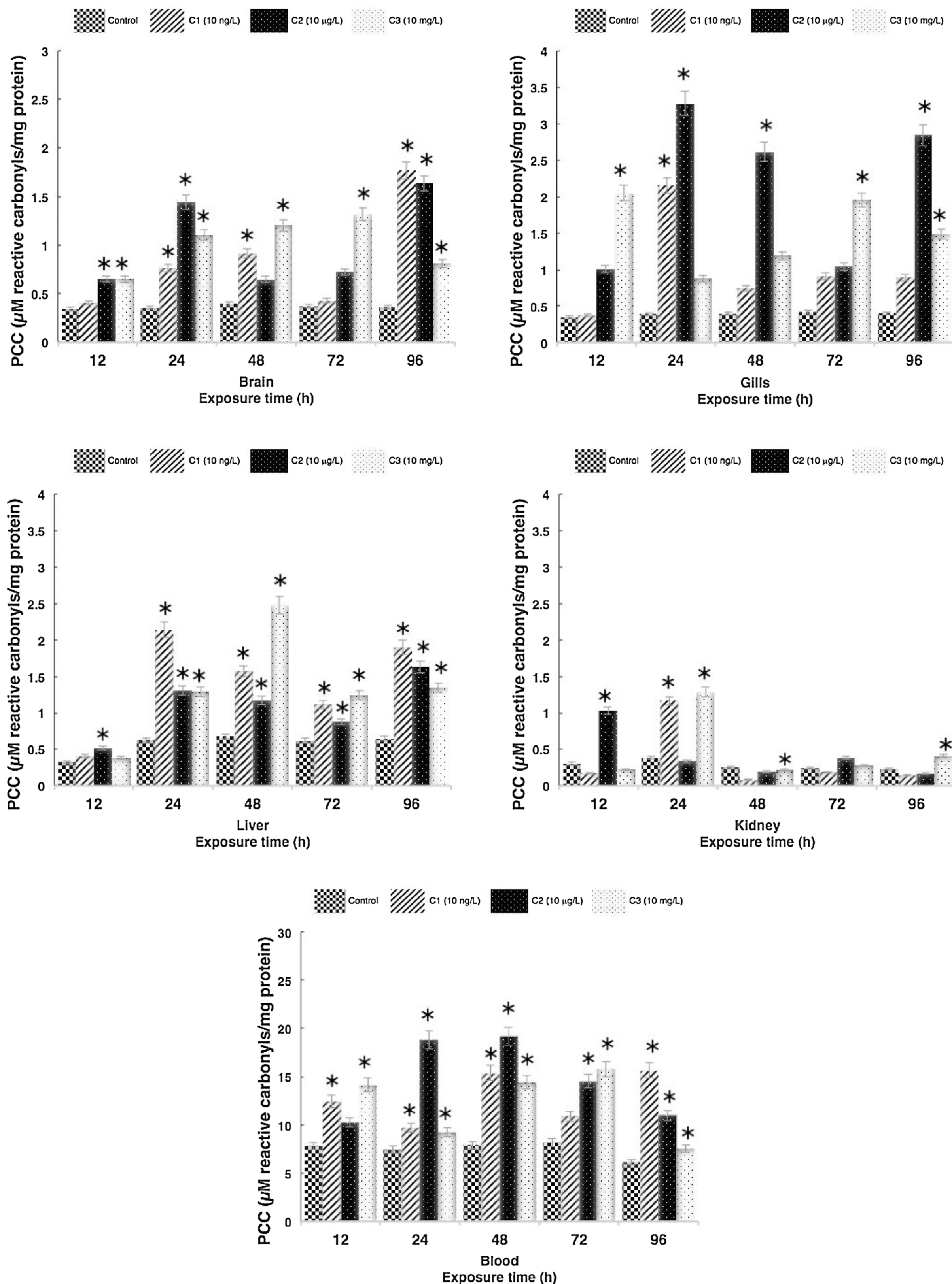

Fig. 3. Protein carbonyl content (PCC) in brain, gill, liver, kidney and blood of *Cyprinus carpio* exposed to three different concentrations of metoprolol (C1, C2 and C3) for 12, 24, 48, 72 and 96 h. Values are the mean of three replicates ± SE. *Significant differences with the control group, ANOVA and Bonferroni ($P < 0.05$).

with production and release of ROS such as the superoxide anion radical (O_2^-) and H_2O_2. In phase I biotransformation, MTP undergoes O-demethylation. During this process, a ternary complex called oxy-cytochrome P450 (O_2-P450-Fe^{2+}-MTP) is formed. This complex can dissociate, yielding O_2^-, with regeneration of the ferric protein P450-Fe^{3+}-MTP and subsequent release of H_2O_2 (Park et al., 1995).

Both O_2^- and H_2O_2 formed in the biotransformation of MTP may be responsible for the oxidative stress found in the different organs of *C.*

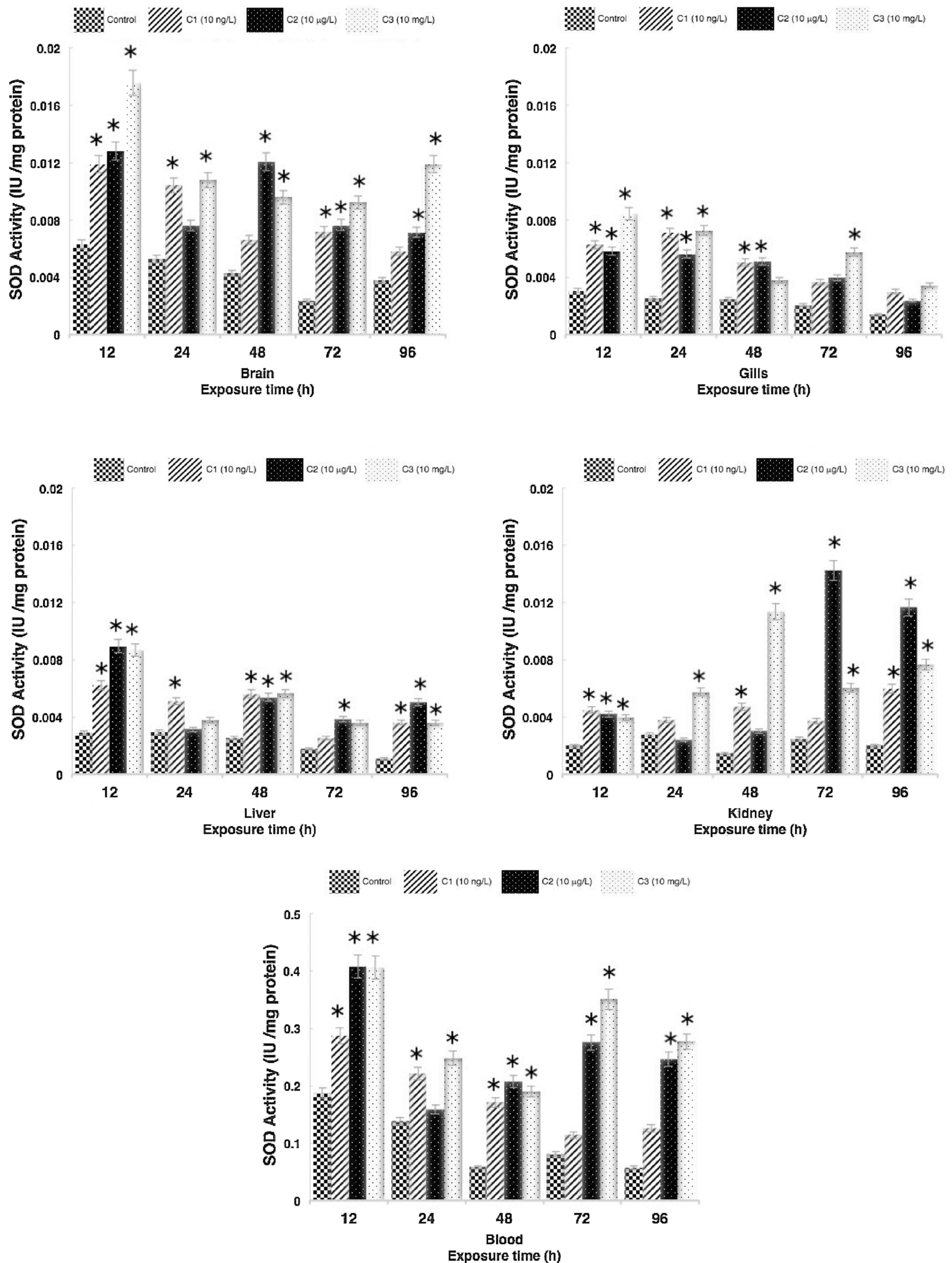

Fig. 4. Superoxide dismutase (SOD) activity in brain, gill, liver, kidney and blood of *Cyprinus carpio* exposed to three different concentrations of metoprolol (C1, C2 and C3) for 12, 24, 48, 72 and 96 h. Values are the mean of three replicates ± SE. *Significant differences with the control group, ANOVA and Bonferroni ($P < 0.05$).

carpio evaluated in our study.

During lipid peroxidation, polyunsaturated fatty acids react with ROS, in particular the hydroxyl radical (HO^-) and O_2^-, through a chain reaction mechanism. This allows formation of hydroperoxides which degrade to low-molecular-weight products such as MDA (determined in this study as LPX) (Wilhelm-Filho et al., 2005); however, there are other degradation products such as hexanal and 4-HNE, which were not quantified in this study. As shown in Fig. 1, HPC increases

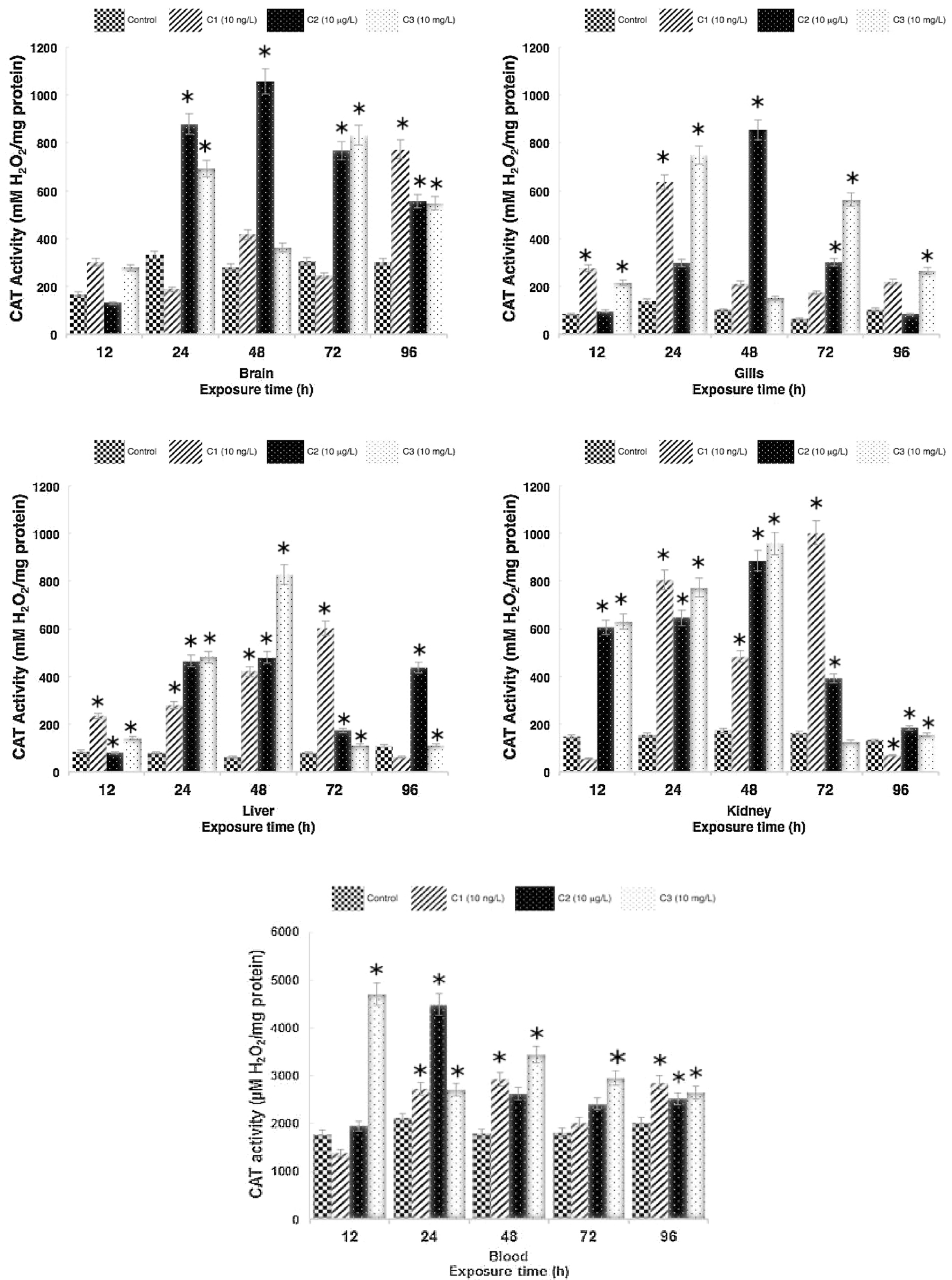

Fig. 5. Catalase (CAT) activity in brain, gill, liver, kidney and blood of *Cyprinus carpio* exposed to three different concentrations of metoprolol (C1, C2 and C3) for 12, 24, 48, 72 and 96 h. Values are the mean of three replicates ± SE. *Significant differences with the control group, ANOVA and Bonferroni ($P < 0.05$).

relative to the control group ($P < 0.05$) occurred in brain, gill, liver, kidney and blood with almost all concentrations at most exposure times. A similar behavior was observed for MDA content (Fig. 2). These results may be explained by O_2^- presence as a result of MTP biotransformation.

The organs evidencing the most relevant increases in HPC and LPX

Table 4
Percentages of increases and decreases of oxidative damage and antioxidant activity comparing 10 ng/L, 10 µg/L and 10 mg/L against the control group.

Oxidative damage					
Biomarkers	Organs	Time (h)	Concentration		
			10 ng L^{-1}	10 µg L^{-1}	10 mg L^{-1}
HPC	BLOOD	12	↑587*	↑75*	↑33
		24	↑12	↑140*	↑16
		48	↑11	↑267*	↑9
		72	↑61*	↑74*	↑6
		96	↑542*	↑235*	↑759*
	LIVER	12	↑114*	↑111*	↑49*
		24	↑139	↑277*	↑2528*
		48	↑286*	↑169*	↑295*
		72	↑39	↑81*	↑121*
		96	↑168*	↑378*	↑331*
	GILL	12	↑185*	↑183*	↑624*
		24	↑533*	↑69*	↑26
		48	↓12	↑295*	↑457*
		72	↓38	↓8	↑290*
		96	↓46*	↑28	↑124*
	BRAIN	12	↓49	↑363*	↑1
		24	↑9	↑36*	↑147*
		48	↑75*	↑324*	↑56*
		72	↑8	↑5	↑191*
		96	↑177*	↓51*	↑16
	KIDNEY	12	↓15	↑109*	↓27
		24	↑139*	↑67	↑279*
		48	↑35	↑512*	↑55
		72	↓1	↑103*	↓13
		96	↑235*	↑3	↑131*
LPX	BLOOD	12	↑83*	↑69*	↑69*
		24	↑82*	↑120*	↑4
		48	↑71*	↑95*	↑72*
		72	↑22	↑121*	↑71*
		96	↑73*	↑40*	↑118*
	LIVER	12	↑92	↑96	↑158*
		24	↑123	↑122*	↑148*
		48	↑115*	↑41	↑127*
		72	↑60	↑191*	↑446*
		96	↑174*	↑91*	↑108*
	GILL	12	↑43	↑26	↑355*
		24	↑226*	↑25	↑124*
		48	↑40	↑436*	↑2
		72	↑16	↑76*	↑342*
		96	↑43	↑325*	↑137*
	BRAIN	12	↑102*	↑145*	↑126*
		24	↑106*	↑275*	↑116*
		48	↑197*	↑118*	↑164*
		72	↑51*	↑16	↑251*
		96	↑44*	↑49*	↑196*
	KIDNEY	12	↑118*	↑101*	↑89*
		24	↑203*	↑83*	↑79*
		48	↑56	↑123*	↑235*
		72	↑78*	↑45	↑54
		96	↑14	↑51	↑109*
PCC	BLOOD	12	↑59*	↑32	↑82*
		24	↑31*	↑154*	↑25*
		48	↑95*	↑143*	↑83*
		72	↑33	↑78*	↑93*
		96	↑156*	↑80*	↑23*
	LIVER	12	↑25	↑59*	↑19
		24	↑241*	↑108*	↑106*
		48	↑133*	↑73*	↑267*
		72	↑79*	↑41*	↑99*
		96	↑192*	↑151*	↑107*
	GILL	12	↑6	↑187	↑487*
		24	↑455*	↑744*	↑126
		48	↑87	↑559*	↑200
		72	↑117	↑151	↑368*
		96	↑123	↑609*	↑271*
	BRAIN	12	↑20	↑91*	↑90*
		24	↑115*	↑305*	↑210*
		48	↑129*	↑62	↑202*
		72	↑15	↑94	↑254*
		96	↑387*	↑351*	↑123*
	KIDNEY	12	↓43	↑231*	↓27
		24	↑205*	↓11	↑237*
		48	↓66	↓22	↓12*
		72	↓23	↑57	↑15
		96	↓37	↓27	↑78*

(*continued on next page*)

Table 4 (*continued*)

Antioxidant activity					
SOD	BLOOD	12	↑54*	↑118*	↑117*
		24	↑59*	↑14	↑79*
		48	↑195*	↑257*	↑228*
		72	↑42	↑242*	↑336*
		96	↑123	↑335*	↑390*
	LIVER	12	↑113*	↑207*	↑198*
		24	↑74*	↑7	↑28
		48	↑120*	↑110*	↑120*
		72	↑44	↑117*	↑102
		96	↑238*	↑371*	↑235*
	GILL	12	↑104*	↑90*	↑175*
		24	↑182*	↑124*	↑189*
		48	↑103*	↑105*	↑53
		72	↑79	↑93	↑181*
		96	↑113	↑69	↑144
	BRAIN	12	↑89*	↑104*	↑180*
		24	↑97*	↑44	↑105*
		48	↑54	↑181*	↑124*
		72	↑202*	↑222*	↑289*
		96	↑52	↑87*	↑212*
	KIDNEY	12	↑124*	↑110*	↑97*
		24	↑37	↓13	↑106*
		48	↑225*	↑108	↑683*
		72	↑51	↑476*	↑145*
		96	↑197*	↑480*	↑281*
CAT	BLOOD	12	↓21	↑10	↑165*
		24	↑29*	↑112*	↑28*
		48	↑63*	↑47	↑92*
		72	↑12	↑33	↑63*
		96	↑41*	↑25*	↑31*
	LIVER	12	↑174*	↓8*	↑64*
		24	↑251*	↑486*	↑505*
		48	↑594*	↑691*	↑1265*
		72	↑639*	↑122*	↑36*
		96	↓42	↑307*	↑3*
	GILL	12	↑223*	↑14	↑152*
		24	↑355*	↑113	↑435*
		48	↑109	↑740*	↑48
		72	↑163	↑356*	↑752*
		96	↑110	↓19	↑154*
	BRAIN	12	↑79	↓22	↑65
		24	↓43	↑165*	↑109*
		48	↑49	↑277*	↑29
		72	↓19	↑151*	↑172*
		96	↑155*	↑84*	↑81*
	KIDNEY	12	↓62	↑314*	↑330*
		24	↑421*	↑318*	↑400*
		48	↑180*	↑413*	↑455*
		72	↑513*	↑140*	↓23
		96	↓46*	↑41*	↑19*

*Significant differences. HPC = hydroperoxide content, LPX = lipid peroxidation, PCC = protein carbonyl content, SOD = superoxide dismutase activity, CAT = catalase activity.

were liver and gill. The liver is the most metabolically active tissue in the body and the site of major processes of biotransformation (Van der Oost et al., 2003). Similarly, in freshwater fish, the gills are known as the organ where oxidative metabolism occurs significantly (Monteiro et al., 2005). The increase in ROS induced by oxidative metabolism in the latter organs may explain the HPC and LPX increases in our study.

ROS produced during MTP biotransformation can affect proteins. ROS can bind to protein sulfhydryl groups, inducing changes in amino acid structure and altering its function, and therefore protein integrity (Parvez and Raisuddin, 2005). On the other hand, O_2^- reacts with nitric oxide from arginine metabolism to produce the reactive nitrogen species peroxynitrite ($ONOO^-$) (Doi et al., 2002; Halliwell, 1997; Jifa et al., 2006), which has a high affinity for protein sulfhydryl groups, favoring their oxidation and nitration in the absence of GSH and inducing mitochondrial dysfunction and in the long run irreversible damage and severe loss of cellular ATP (Jaeschke and Lemasters, 2003). These findings agree with our results, which showed a significant increase in PCC in all organs evaluated at most exposure times.

Decreases at 96 h with respect to the different exposure times in the biomarkers of damage (HPC, LPX and PCC) can be explained by the action of antioxidant enzymes to counteract the damage. Antioxidant defenses are induced by diverse environmental as well as xenobiotic factors under pro-oxidant conditions (Cheung et al., 2001). Antioxidant levels initially increase in order to offset the oxidative stress, but prolonged exposure leads to their depletion (Bebianno et al., 2005; Cheung et al., 2001). This behavior was observed in antioxidant enzyme activities (SOD and CAT).

The antioxidant system is mediated by a cascade of antioxidant enzymes that can sequester ROS and convert them to less toxic and reactive species. This enzyme group includes SOD and CAT. The former is the first mechanism of antioxidant defense and the main enzyme responsible for dismutation of the superoxide anion to yield H_2O_2, and it is this substrate of CAT that degrades H_2O_2 to H_2O and O_2 (Orsi and Leese, 2001; Van der Oost et al., 2003).

In the present study, SOD and CAT activity increased significantly in all organs with respect to the control group ($P < 0.05$) at the different exposure times. In blood, liver and kidney, this increase was more pronounced from 12 h on. These results concur with those of Contardo-Jara et al. (2010), who showed that SOD and CAT activity increased in digestive gland of *D. polymorpha* exposed to 537.54 µg MTP/L. The

latter authors determined up-regulation of the mRNA of genes encoding heat shock proteins, which presupposes protein damage and the need for repair processes, such as increased antioxidant enzyme levels. They also reported that MTP was highly bioaccumulated at environmentally relevant concentrations, as was also observed in our study.

Results of the present study indicate that the oxidative biotransformation of MTP is crucial in explaining the increases in the levels of indicators of cellular oxidation and antioxidant defense in *C. carpio* and that, although this pharmaceutical is moderately lipophilic, both the parent molecule and its metabolite O-DMM are able to bioconcentrate in the different organs assayed. However, the results show that there is no relationship of all times and concentrations with respect to the observed damage (Table 5, in supplement material). These results are consistent with those reported by Gröner et al. (2015), who investigated the expression levels of genes encoding key enzymes of the xenobiotic metabolism and excretion pathways concerning biotransformation phases I, II and III and of the estrogenic biomarker vitellogenin in primary hepatocytes isolated from male Nile tilapia (*Oreochromis niloticus*) after exposure to diclofenac and metoprolol. The authors conclude that there is not a concentration-dependency for metoprolol.

5. Conclusions

MTP started to degrade to O-DMM during the first 12 h of exposure. *C. carpio* take up MTP and O-DMM from 12 h of exposure onward. MTP and O-DMM were responsible for the increases in HPC, LPX, PCC and the antioxidant activity of SOD and CAT in all organs evaluated. Gill and liver were the most affected organs, while brain was the least susceptible to these compounds. The presence of pharmaceuticals, such as MTP and O-DMM, in the environment may constitute a risk in water bodies for aquatic species as *Cyprinus carpio*.

Conflicts of interest

None.

Ethical approval

This protocol was reviewed and approved by the Bioethics Committee of the Universidad Autónoma del Estado de México (UAEM) to ensure that it was carried out in accordance with institutional standards for the care of animal subjects. Provisions in the official Mexican norm on breeding, care and use of laboratory animals (NOM-062-ZOO-1999) were also taken into account.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.aquatox.2018.02.012.

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