Comparative Biochemistry and Physiology, Part C 240 (2021) 108910

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Diclofenac and caffeine inhibit hepatic antioxidant enzymes in the freshwater fish *Astyanax altiparanae* (Teleostei: Characiformes)

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ARTICLE INFO

Keywords:
Drugs
Genotoxicity
Lipoperoxidation
Oxidative stress
Water pollution

ABSTRACT

Although concentrations of pharmaceutical compounds in aquatic ecosystems are low, they can cause toxic effects on organisms. The aim of this study was to evaluate the effects of diclofenac (DCF), a non-steroidal anti-inflammatory drug, and caffeine (CAF), a central nervous system stimulant, both alone or combined, in *Astyanax altiparanae* males under acute exposure (96 h), measuring neurotoxicity biomarkers, antioxidant response and damage at biochemical and cellular levels. DCF concentration in water, separated and combined, was 3.08 mg L^{-1} and that of CAF was 9.59 mg L^{-1}. To assess neurotoxicity, brain and muscle acetylcholinesterase (AChE) activities were measured. To evaluate oxidative stress, the enzymatic activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and glutathione S-transferase (GST), as well as lipoperoxidation (LPO), were analyzed in liver and gills. Activity of hepatic cyclooxygenase (COX) was also evaluated. Genotoxicity was assessed in blood using comet assay and micronucleus test, as well as nuclear abnormalities. DCF and CAF, alone or combined, had neither effect on AChE activity, nor in the activity of SOD, CAT, GPx and GST in gills. In liver, DCF inhibited SOD and GPx activity, CAF inhibited CAT activity, the mixture inhibited SOD and GST activity; although only fish exposed to CAF showed increased hepatic LPO. Under these experimental conditions, no effect on COX activity was observed, nor cytotoxic and genotoxic damage. The most pronounced effects were caused by the drugs separately, since both compounds altered the enzymes, but only CAF triggered LPO, showing more harmful effects.

1. Introduction

Pollution of freshwater bodies is a problem that has attracted the interest of the scientific community for several decades. In recent years compounds from the pharmaceutical industry have gained special interest and have been included in the category of contaminants of emerging concern, as these compounds, that were not previously considered potentially toxic for the environment, are now known for their estrogenic, genotoxic, carcinogenic and teratogenic effects in aquatic communities (Tejada et al., 2014; Bing-Shu et al., 2017).

Among the main pharmaceutical compounds present in water bodies are the non-steroidal anti-inflammatory drugs (NSAIDs), such as diclofenac (DCF) which has the main function of inhibiting the enzyme cyclooxygenase (COX), which acts in the synthesis of prostaglandins involved in inflammatory processes (Hoeger et al., 2005; Bing-Shu et al., 2017). Some studies have shown that DCF is one of the most consumed anti-inflammatory drugs worldwide, and one of the most representative in the aquatic environment (Bing-Shu et al., 2017). In addition, even in water treatment plants, it has been observed that the DCF removal efficiency is only 40% (Almeida and Weber, 2005; Cherik et al., 2015). Considering the above, several studies have classified DCF as one of the most toxic pharmaceutical compound for aquatic organisms at all trophic levels (Bing-Shu et al., 2017), causing cytological changes in rainbow trout (*Oncorhynchus mykiss*) (5, 20, 100 and 500 µg L^{-1}) (Schwaiger et al., 2004); oxidative stress in common carp (*Cyprinus carpio*) (7.098 mg L^{-1}) (Islas-Flores et al., 2013) and trahira

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https://doi.org/10.1016/j.cbpc.2020.108910
Received 7 July 2020; Received in revised form 28 August 2020; Accepted 27 September 2020
Available online 10 October 2020

(*Hoplias malabaricus*) (supplied by food: 0.2, 2 and 20 μg kg^{-1}) (Guiloski et al., 2015); changes in the embryonic development of zebrafish (*Danio rerio*) (1.5, 2.9, 5.9, 11.9 and 23.7 mg L^{-1}) (Van der Brandohf and Montforts, 2010), together with endocrine disrupting effect in the yellow-tailed lambari (*Astyanax altiparanae*) (4.4 mg L^{-1}) (Godoi et al., 2020). Thus, considering the evidence of its high toxicity, the European Commission (2012) included DCF as a priority hazardous substance, being 100 ng L^{-1} the maximum concentration allowed in freshwater (Acuña et al., 2015).

In addition to NSAIDs, other drugs ubiquitous in water bodies are stimulants such as caffeine (CAF). CAF is used as a cardiac, brain and respiratory stimulant, being commonly consumed in food products such as coffee, tea and chocolate (Moore et al., 2008; Lee and Wang, 2015). Within the main mechanisms of action, CAF is antagonist of adenosine receptors; it is involved in mobilization of intracellular calcium and inhibition of phosphodiesterase (Cappelletti et al., 2015; Nehlig et al., 1992). Presence of CAF in water bodies has also been reported due to its high solubility and difficult depuration in water treatment plants (Oliveira et al., 2015). However, information about the effects of CAF pollution on the aquatic ecosystems is still scarce (Moore et al., 2008). Some recent studies have demonstrated bioaccumulation of CAF in the mosquito fish (*Gambusia holbrooki*) (1.3 ng g^{-1}) (Wang and Gardinali, 2012), interference of CAF in the development of embryos and larvae of the fathead minnow (*Pimephales promelas*) (50 mg L^{-1}) (Moore et al., 2008) and the medaka fish (*Oryzias latipes*) (10 and 100 mg L^{-1}) (Lee and Wang, 2015), as well as CAF as an endocrine disruptor in the yellow-tailed lambari (*A. altiparanae*) (12.8 mg L^{-1}) (Godoi et al., 2020).

The presence of both drugs has been widely reported worldwide in surface water bodies, with average concentrations of 21 to 722 ng L^{-1} for DCF (Acuña et al., 2015), and between 2 and 1600 ng L^{-1} for CAF (Capolupo et al., 2016). In Brazil, presence of both drugs in water bodies has been also reported (Starling et al., 2019) with concentrations of up to 394.5 ng L^{-1} of DCF (Almeida and Weber, 2005) and 27,386 ng L^{-1} of CAF (Shihomatsu et al., 2017) in reservoirs in the São Paulo Metropolitan Region (MRSP). The effects of DCF and CAF have been demonstrated in some teleost species, however little information is available on the toxicological effects of the mixture of these two compounds. This is relevant considering that aquatic organisms are constantly exposed to mixtures of pollutants, which could have a completely different effect than the compounds alone (Shi et al., 2019). In fish, it has been reported so far that the mixture of DCF and CAF causes an increase in the gonadosomatic index (GSI) in adult males of *A. altiparanae*. These same species, when exposed to these compounds separately, showed a decrease in plasma concentration of 17β-estradiol; DCF also triggered a reduction of plasmatic testosterone (Godoi et al., 2020) showing that the separated and combined effects differ in endocrine responses, as CAF and DCF combined did not show endocrine disrupting effects.

In the present study, *A. altiparanae* was used due to its plasticity under different environmental conditions, its suitable size for maintenance in aquaria and easily handled in the laboratory. In fact, this species has been previously used in other ecotoxicological studies (Kida et al., 2016; Abdalla et al., 2019; Pinheiro et al., 2019, 2020; Godoi et al., 2020). In addition, this species is widely distributed throughout South America (Garutti and Britski, 2000), being abundant and one of the most representative in the RMSP reservoirs, where other studies have been carried out (Gomes et al., 2015, 2016; Tolussi et al., 2018; Marques et al., 2020). Besides, these urban reservoirs have been impacted by intense anthropogenic actions, being caffeine and diclofenac two of the main drugs found in water in these environments (López-Doval et al., 2017; Quadra et al., 2017). Based on the above, the aim of this study was to evaluate the effects of diclofenac (DCF), a non-steroidal anti-inflammatory drug, and caffeine (CAF), a central nervous system stimulant, both alone or combined, in *Astyanax altiparanae* males under acute exposure (96 h), measuring neurotoxicity biomarkers, antioxidant response and damage at biochemical and cellular levels.

2. Materials and methods

2.1. Chemicals

Sodium Diclofenac (D6899) (purity ≥98%) and caffeine (C0750) (purity ≥98%) were obtained from Sigma-Aldrich. Solutions were prepared by diluting the drugs at the time of use in deionized water, according to the manufacturer's instructions, depending on the concentrations required for the different experimental groups.

2.2. Experimental design

Adult males of *A. altiparanae* (W: 14.76 ± 6.70 g; TL: 10.27 ± 1.46 cm) were provided by *Estação de Hidrobiologia e Aquicultura de Paraibuna* (23°24′53.1″S 45°35′59.5″W) in Paraibuna City (São Paulo, Brazil), and transported to the laboratory where they were acclimated before the beginning of the experiments (water temperature: 25.3 ± 0.26 °C; dissolved oxygen: 6.4 ± 0.2 mg L^{-1}; pH: 7.6 ± 0.19 and photoperiod: 12:12). *A. altiparanae* adults present a sexual dimorphism (males exhibit roughness in the anal fin) and they were chosen due to the lower metabolic investment compared to females. The fish were fed daily ad libitum with extruded feed (32% crude protein) and water was renewed every 48 h.

For the acute toxicity test a sublethal concentration of 10% of the LC_{50} value of each drug for *A. altiparanae* (3.08 mg L^{-1} for DCF and 9.59 mg L^{-1} for CAF) (Godoi et al., 2020) was used. Fish were randomly divided into 120 L aerated aquaria in four groups (n = 12 in each group): control (CTR), diclofenac (DCF: 3.08 mg L^{-1}), caffeine (CAF: 9.59 mg L^{-1}), and the mixture of diclofenac and caffeine (DCF: 3.08 mg L^{-1} + CAF: 9.59 mg L^{-1}). The acute toxicity test was performed for 96 h of exposure and water renewal (75%) was carried out every 48 h in order to maintain the drug concentrations, following the results of the decay test for each drug in our previous study (Godoi et al., 2020). Physical-chemical parameters of water were monitored daily (temperature: 25.43 ± 0.13 °C; pH: 7.49 ± 0.02 and dissolved oxygen: 6.34 ± 0.07 mg L^{-1} and photoperiod: 12:12). All procedures were approved by the Ethics Committee on Animal Use (CEUA) of the Institute of Biosciences, University of São Paulo (USP) (Protocol number 275/2017).

2.3. Drug concentration analysis

The analysis of drug concentration in water samples was conducted at the Center for Applied Mass Spectrometry of the Institute of Energy and Nuclear Research at University of São Paulo. Water samples were collected (0 h and 48 h of exposure - after water renewal) on the same spot of the aquarium and filtered with 45 μm filters (Sartorius Stedim Biotech) and stored in amber flasks at 10 °C. Concentration of drugs in water was analyzed by LC–MS/MS technique by a 1260 (Agilent Technologies, USA) Luna C18 HST (2) (100 × 2 mm; 2.5 μm) (Phenomenex) column at 35 °C combined with a 3200QTRAP mass spectrometer (MS-MS) (ABSciex). Volume sample of injection was 5 μL, eluent flux rate was 220 μL min^{-1} and the mobile phase was 0.1% formic acid (Sigma-Aldrich LC–MS Grade) in ultrapure water (solvent A) and 0.1% formic acid in acetonitrile (J.T. Baker LC–MS Grade) (solvent B). For solvent A the eluent equilibrium was 80% and for solvent B was 20% in 2.30 min run. Analytes were detected and quantified using ESI ionization and Multiple Reaction Monitoring (MRM) mode. Linearity for both drugs were r = 0.99, detection limit was 0.054 μg L^{-1} for DCF and 1.5 μg L^{-1} for CAF and quantification limit for DCF was 0.18 μg L^{-1} and for CAF was 5.1 μg L^{-1}. Data were recorded and processed using Analyst® 1.5.2 (ABSciex). The effective concentration for DCF was 4.35 ± 0.40 mg L^{-1}, for CAF was

11.63 ± 0.62 mg L^{-1}, and in the mixture treatment (DCF + CAF) it was 3.98 ± 0.23 and 12.23 ± 0.03 mg L^{-1}, respectively.

2.4. Sample collection

At the end of exposure, fish were anesthetized by immersion in water with benzocaine (0.1%) previously diluted in 10 mL of ethanol. After the opercular movement ceased (less than 2 min), blood samples were collected by puncturing the caudal vasculature using heparinized syringes and needles, and 10 µL were stored in microtubes with 1 mL of cryopreservation buffer (250 mM sucrose, 40 mM trisodium citrate, 5% dimethyl sulfoxide (DMSO), pH 7.6, adjusted with 1 M of citric acid), and maintained in liquid nitrogen until the processing for the Comet assay (Evrard et al., 2010).

In parallel, smears were performed with 5 µL of blood for the analysis of the micronucleus and nuclear abnormalities test. Concomitantly, morphometric and weight data were recorded and afterwards, fish were euthanized by the section of the spinal cord at the operculum level (National Research Council, 2011). After dissection, brain, gill filaments, liver and white muscle were collected and kept at −80 °C until processing.

2.5. Enzymes activities analysis

In order to measure the activity of the oxidative stress enzymes (superoxide dismutase - SOD, catalase - CAT, glutathione peroxidase - GPx, and glutathione S-transferase - GST), samples of liver and gill filaments were homogenized in potassium phosphate buffer (0.1 M; pH 7.0; 1:10 w v^{-1} for liver and 1:5 w v^{-1} for gills), centrifuged (15,000 ×*g*, 20 min, 4 °C) to use the supernatants. SOD activity was quantified according to the method described by McCord and Fridovich (1969), using a 550 nm spectrophotometer (25 °C). The SOD present in the sample inhibited the reduction of cytochrome C provided by the superoxide anion of the xanthine/xanthine oxidase system. The result was expressed in U of SOD mg of protein^{-1}, which represents the amount of SOD that promotes 50% inhibition of the reduction rate of cytochrome C. GPx activity was quantified according to the method described by Hopkins and Tudhope (1973), estimating GPx activity through the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of H_2O_2, using the spectrophotometer at 340 nm (25 °C). CAT activity was quantified according to the method described by Beutler (1975), through the rate of H_2O_2 decomposition by the enzyme. The reading was done on a spectrophotometer at 240 nm (25 °C). GST activity was quantified according to the method described by Habig et al. (1974). The complexation of GSH activity with 1-chloro-2,4-dinitrobenzene (CDNB) allows the GSH concentration to be estimated, with a spectrophotometer reading at 340 nm (25 °C).

To assess neurotoxicity, brain or muscle samples were homogenized in potassium phosphate buffer (0.1 M; pH 7.5; 1:10 w v^{-1}) and centrifuged (13,000 rpm, 20 min, 4 °C). The supernatant was used to analyze acetylcholinesterase (AChE) activity following the method described by Ellman et al. (1961) and modified by Alves-Costa et al. (2007). AChE activity was evaluated in a spectrophotometer at 415 nm (25 °C) and expressed in nmol per minute per mg of protein.

Total protein concentration was quantified in all tissues (Lowry et al., 1951) to normalize the analyzed enzymes (with the exception of COX) using bovine serum albumin as standard, at 660 nm. The activity of the cyclooxygenase enzyme (COX) in the liver was analyzed with the Cayman Chemical colorimetric kit (No. 760151), according to the manufacturer's recommendations (in duplicate). The activity was expressed as nmol min^{-1} mL^{-1}. All measurements were carried out in a microplate reader (SpectraMax 190, Molecular Devices).

2.6. Lipoperoxidation analysis

Liver and gills lipoperoxidation (LPO) were analyzed using the xylenol ferrous/orange oxidation (FOX) method adapted by Hermes-Lima et al. (1995), with spectrophotometer reading at 580 nm. This method evaluates LPO in the intermediate phase of the process, measuring the formation of lipid hydroperoxides using an assay based on the formation of a Fe (III) xylenol orange complex. The method is based on the oxidation of Fe(II) by lipid hydroperoxides at acidic pH in the presence of the Fe(III)-complexing dye, xylenol orange. The formation of the Fe (III) xylenol orange complex reflects a chemical amplification of the original level of lipid hydroperoxides present in tissue extracts. The lipid hydroperoxides were expressed as CHP (cumene hydroperoxide equivalents) g^{-1} wet weight.

2.7. Genotoxicity analyzes

The Comet assay (single-cell gel electrophoresis) was performed according to the method described by Singh et al. (1988). 10 µL of the preserved blood was added to 120 µL of low melting point agarose for homogenization. Then, two slides with agarose (1% in PBS) were prepared per sample and subjected to the processes of: 1) lysis for 1 h at 4 °C protected from light in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1% TRITON X-100, 0.01 M sodium lauryl sarcosinate); 2) DNA denaturation for 30 min in electrophoresis buffer in the dark (300 mM NaOH, 100 mM EDTA, pH 13); 3) electrophoresis for 20 min at 300 mA and at 1 V per centimeter in length of the plate; 4) washing with neutralization solution three times (0.4 M Tris). Subsequently, slides were fixed in absolute ethanol for 10 min. At the time of analysis, GelRed® Biotium (Sigma-Aldrich) was used to stain and 100 cells per slide were counted under inverted fluorescence microscope (400 x) (Leica DMi8). The classification of each cell was performed according to Kobayashi et al. (1995): class 0 = no apparent damage; class 1 = tail length less than the diameter of the nucleus; class 2 = tail length equal to the nucleus diameter; class 3 = tail length greater than the diameter of the nucleus. Scores for each experimental group were calculated by multiplying the number of nucleoids seen in each class by the class value (0, 1, 2 or 3). Finally, the scores of all individuals within the treatment were added and divided by the number of individuals, resulting in the average score.

The micronucleus test was performed according to the method described by Al-Sabti and Metcalfe (1995) and the abnormality test according to Carrasco et al. (1990). For both analyzes, after 24 h of blood smears (duplicate per animal), slides were fixed in absolute methanol for 10 min and stained with Giemsa (10%). 1000 erythrocytes were examined per slide under a light microscope (1000×) (microscope Leica DM1000, photographic Leica DFC295 camera and image capture Leica Application Suite Professional software, LAS V3.6).

2.8. Statistical analysis

All data were submitted to normality tests (Shapiro-Wilks) and homogeneity of variance (Bartlett's test). To assess the differences in physiological responses between experimental treatments, the one-way ANOVA test was used, followed by the Tukey test for parametric data (AChE in muscle, SOD and LPO in gills, and SOD, GPx, CAT and GST in liver) or the Kruskal-Wallis test followed by the Dunns test for non-parametric data (AChE in brain, GPx, CAT and GST in gills, LPO and COX in liver, Comet assay, micronucleus test and nuclear abnormalities). In all cases, a significance level of $P < 0.05$ was considered to be statistically significant. GraphPad Prism 5.01 for windows (GraphPad Software Inc., San Diego, CA) was used for all statistical analyzes.

3. Results

3.1. Oxidative stress biomarkers

In the liver, exposure to drugs showed a decrease in the activity of antioxidant enzymes. SOD activity was reduced in fish exposed to DCF

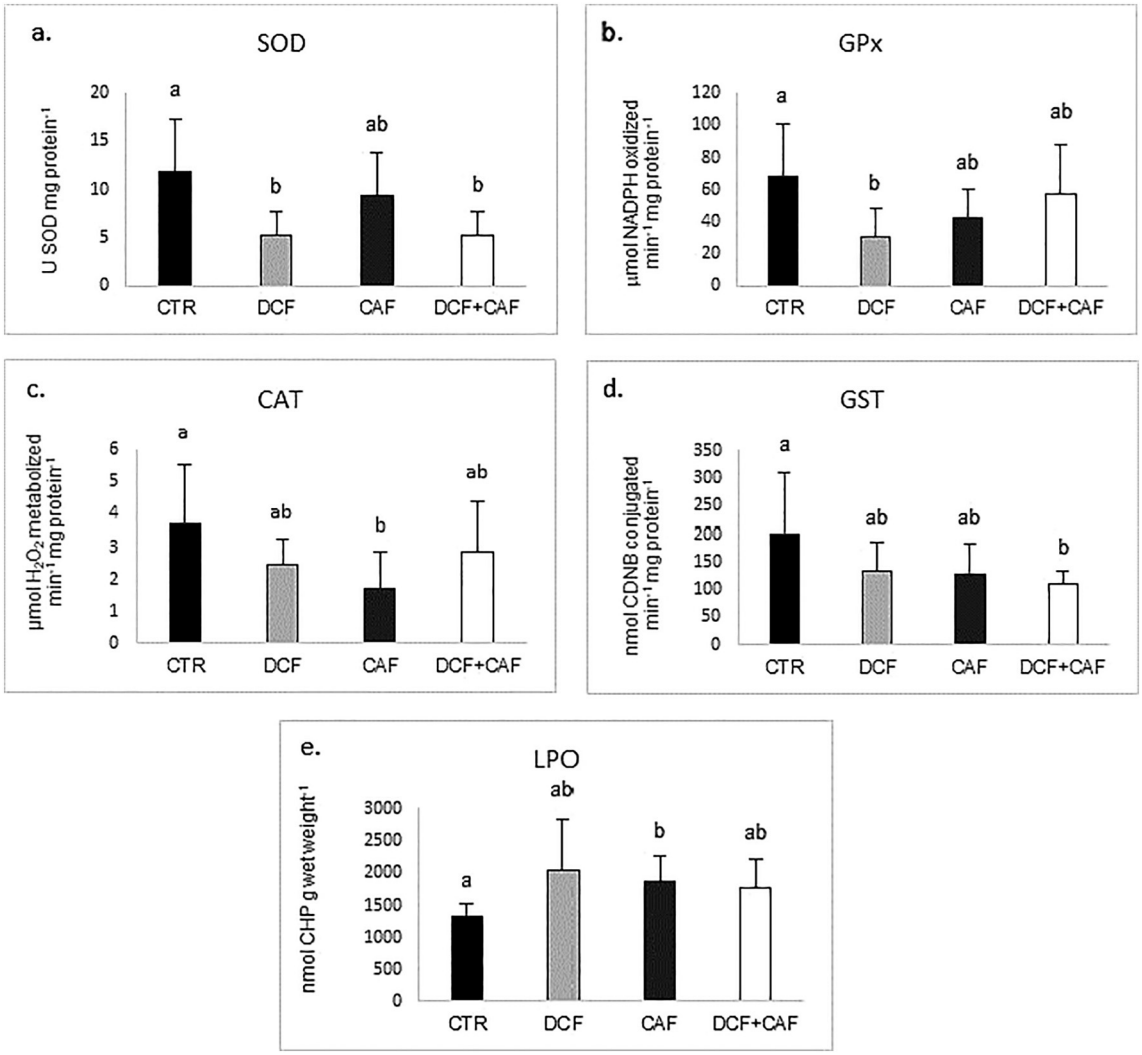

Fig. 1. Oxidative stress biomarkers in the liver of *A. altiparanae* males under different treatments after 96 h of exposure. CTR (control), DCF (diclofenac), CAF (caffeine), DCF + CAF (diclofenac + caffeine). Superoxide Dismutase (SOD) (a), Glutathione Peroxidase (GPx) (b), Catalase (CAT) (c), Glutathione S-Transferase (GST) (d) and Lipoperoxidation (LPO) (e). [ab] Different letters represent statistically significant differences between treatments (ANOVA, $P < 0.05$). Values are expressed as mean ± SD of the mean. n = 12.

alone and combined with CAF (Fig. 1a, P = 0.007), GPx activity was reduced in fish exposed to DCF (Fig. 1b, P = 0.033), CAT activity was reduced in fish exposed to CAF (Fig. 1c, P = 0.025), while GST activity was reduced in fish exposed to the mixture of both drugs (Fig. 1d, P = 0.043). Despite the reduction in the activity of these enzymes, damage to the liver membranes was only observed in fish exposed to CAF, when compared to CTR group (Fig. 1e; P = 0.01).

Regarding the activity of the enzymes in the gills, no effects of the pharmaceutical compounds were observed on the activity of SOD (P = 0.76), GPx (P = 0.57), CAT (P = 0.08) and GST (P = 0.80) (Fig. 2a–d), and the LPO results showed less damage to the membranes of fish exposed to DCF, compared to CTR group (Fig. 2e; P = 0.016).

3.2. AChE and COX activities

In the present study, no statistically significant differences were observed in the activity of the AChE enzyme in the brain (P = 0.99) and in the muscle (P = 0.70) of *A. altiparanae* adult males under the different experimental treatments (Table 1). Likewise, the activity of cyclooxygenase (COX) in the liver was not altered (Table 1; P = 0.13).

3.3. Genotoxicity biomarkers

According to the results of the genotoxicity analysis, it was not possible to observe genotoxic damage associated with acute exposure to CAF and DCF, using the comet assay (P = 0.61), the micronucleus and nuclear abnormalities tests as biomarkers (P = 0.62) (Table 2).

4. Discussion

In the present study, it was possible to show that DCF and CAF, combined or separated, decreased the activity of some antioxidant system enzymes in the liver of *A. altiparanae* adult males exposed to these drugs for 96 h, which could compromise the health of the organisms. However, only CAF by itself caused hepatic LPO. The activity of gill antioxidant enzymes, on the other hand, did not show any significant alteration, though the organisms exposed to DCF separately showed a lower LPO than CTR fish. In addition, under the experimental conditions of this study, both drugs did not trigger genotoxic or neurotoxic effects, or changes in COX activity.

The liver was the organ where the effects of the evaluated drugs were mainly observed, which makes sense since it is the organ where free radicals are generated at the highest rate, for being the primary site

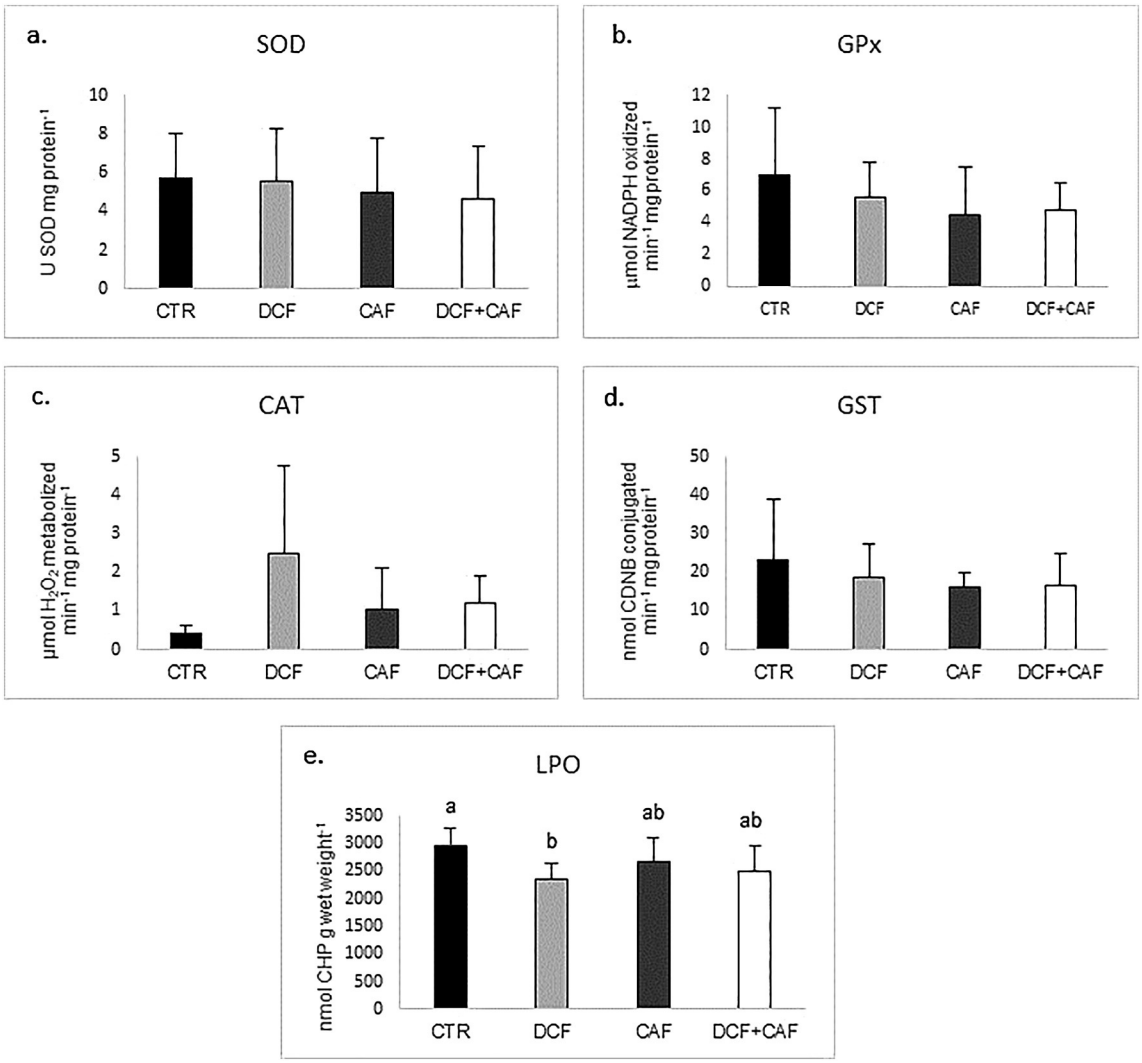

Fig. 2. Oxidative stress biomarkers in the gills of *A. altiparanae* males under different treatments after 96 h of exposure. CTR (control), DCF (diclofenac), CAF (caffeine), DCF + CAF (diclofenac + caffeine). Superoxide Dismutase (SOD) (a), Glutathione Peroxidase (GPx) (b), Catalase (CAT) (c), Glutathione S-Transferase (GST) (d) and Lipoperoxidation (LPO) (e). [ab] Different letters represent statistically significant differences between treatments (ANOVA, $P < 0.05$). Values are expressed as mean ± SD of the mean. n = 12.

Table 1
Acetylcholinesterase (AChE) activity in the brain and muscle, and Cyclooxygenase (COX) activity in the liver of *A. altiparanae* males under different treatments after 96 h of exposure.

	Brain AChE (nmol min^{-1} mg protein^{-1})	Muscle AChE (nmol min^{-1} mg protein^{-1})	Liver COX (nmol min^{-1} mL^{-1})
CTR	53.25 ± 14.31	19.57 ± 5.63	28.20 ± 7.10
DCF	52.77 ± 11.78	18.03 ± 5.79	30.30 ± 8.60
CAF	56.94 ± 16.11	17.10 ± 5.23	40.30 ± 3.70
DCF + CAF	52.36 ± 11.20	18.46 ± 3.14	31.80 ± 8.20

CTR (control), DCF (diclofenac), CAF (caffeine), DCF + CAF (diclofenac + caffeine). Values are expressed as mean ± SD of the mean. n = 12. P = 0.99 (brain AChE); P = 0.70 (muscle AChE) and P = 0.13 (liver COX).

Table 2
Genotoxicity biomarkers in *A. altiparanae* males in different treatments after 96 h of exposure.

	Comet assay (Score of DNA damage)	ENAs frequency (%)	MN frequency (%)
CTR	78.82 ± 12.29	0.06 ± 0.06	0
DCF	85.00 ± 9.05	0.05 ± 0.06	0
CAF	83.84 ± 4.48	0.06 ± 0.07	0
DCF + CAF	83.53 ± 5.99	0.11 ± 0.10	0

CTR (control), DCF (diclofenac), CAF (caffeine), DCF + CAF (diclofenac + caffeine). ENAs (Erythrocyte Nuclear Abnormalities), MN (Micronucleus). Values are expressed as mean ± SD of the mean. n = 12. P = 0.61 (comet assay) and P = 0.62 (ENAs frequency).

of oxidative reactions (Atli et al., 2016). It was in this organ that the greatest effects of drugs on enzymes activity were observed. In general, the activity of antioxidant enzymes was reduced in fish exposed to DCF (with the exception of CAT), contrary to expectations, since when organisms are exposed to toxic agents, the production of ROS may increase as a result of the biotransformation process of these compounds. Therefore, it is expected for the organism to increase its defense system activity (Lesser, 2012; Atli et al., 2016), as has been reported by other authors in different fish species. Islas-Flores et al. (2013) reported an increase in the activity of SOD, CAT and GPx in the liver and gills of common carp (*C. carpio*) as a consequence of acute exposure to DCF (7098 mg L^{-1}). The former was expected by the authors because DCF metabolism favors formation of the O_2^- anion, which activates SOD activity to transform this anion into H_2O_2, and as a consequence,

increase CAT and GPx activity, which participate in the dismutation of this compound into H_2O. Likewise, in trahira (*H. malabaricus*)-fed *Astyanax* sp. previously injected with 2 and 20 μg kg^{-1} of DCF, an increase in SOD and GPx activities were observed (Guiloski et al., 2015), proving an activation of the antioxidant system as a result of the generation of ROS. However, the inhibition observed in our study can be explained by the fact that several metabolites of DCF have the ability to bind to proteins and inhibit the activity of these enzymes (Islas-Flores et al., 2013), which probably occurred in *A. altiparanae* exposed to DCF. In murine, exposure to 10 or 100 mg kg^{-1} of DCF has already been reported as a higher level of oxidized proteins, especially in the heart and liver of these fish, thus generating an imbalance in the proteostasis (Ghosh et al., 2016). Nevertheless, despite the inhibition of the antioxidant system, acute exposure to DCF, alone or combined, did not trigger damage to lipid membranes, contrary to what was observed with exposure to CAF.

Fish exposed to CAF showed a decrease in the activity of CAT, which may have reflected in the increase in LPO considering that CAT acts mainly against more severe damage compared to GPx (Matés, 2000; Stepanova et al., 2013; Atli et al., 2016). In rats, it has already been reported that the supply of 6 mg kg^{-1} of CAF suppresses hepatic CAT activity (Barcelos et al., 2014). However, there are few studies on the effect of this drug on fish's antioxidant system. Although studies on the exposure of aquatic organisms to CAF are scarce, Santos-Silva et al. (2018) evaluated the effects of exposing streaked prochilod (*Prochilodus lineatus*) to CAF (0.3, 3 and 30 μg L^{-1}) for 24 and 168 h and they did not observe changes in GST activity in the brain and liver, and there was no change in LPO either, different to what was observed in the present study. The results of the hepatic antioxidant enzymes and LPO showed a more toxic effect of the compounds separately than mixed; being evident a more pronounced damage to the exposure to CAF, since the fish of this treatment presented hepatic LPO.

The studies carried out so far have emphasized that the effects of xenobiotic compounds on aquatic organisms cannot be generalized, as not all species exhibit the same responses when exposed to certain drugs, which suggests differences in tolerance between species. In addition, effects would also depend on the analyzed tissue, concentration and exposure time. In Nile tilapia (*Oreochromis niloticus*) as concentration (0.17, 0.34 and 0.68 mg L^{-1}) and time of exposure (15, 30, 45 and 60 days) to DCF increased, the activity of SOD, GPx and GST also increased, possibly as a result of the bioaccumulation of the compound (Pandey et al., 2017). On the other hand, in silver catfish (*Rhamdia quelen*) exposed to DCF for 96 h (0.2, 2 and 20 μg L^{-1}), no changes in liver enzyme activity was observed at any concentration, but an increase in SOD activity was observed in the head kidney using the two highest concentrations (Ghelfi et al., 2016). In the present study, it was also possible to observe a difference in the responses of the antioxidant system between the two evaluated organs, showing a negative effect on the hepatic antioxidant system, while the gills did not change, even being the first organ to be in contact with the xenobiotic compounds. In *A. altiparanae*, DCF exposure triggered a lower value of gills LPO compared to fish from the CTR group, that can be explained by the trend to the increased activity of branchial CAT, which, as mentioned earlier, acts against severe damages (Matés, 2000; Stepanova et al., 2013; Atli et al., 2016).

Another biomarker used in the present study for neurotoxicity was the activity of the AChE enzyme, whose main function is to catalyze the hydrolysis of acetylcholine thus allowing the transmission of nerve impulses. In the present study, DCF and CAF had no effect on AChE activity in *A. altiparanae*, possibly due to the short duration of exposure, but it could be possible that the evaluated pharmaceuticals do not have a neurotoxic effect on fish. Generally, the activity of this enzyme is susceptible to changes resulting from exposure to pesticides (Colin et al., 2016); however, it must be considered that the effects that each compound has on a given organism will depend on many variables, including sensitivity of the species and time and concentration of exposure. Oliveira dos Santos et al. (2020) observed an increase in muscle AChE activity in the dusky million fish (*Phalloceros harpagos*) under acute exposure to propanolol, a β-blocking agonist used in cardiac pathologies. This effect was considered atypical by the authors, considering that propranolol generally inhibits AChE activity in several species.

Although the main function of DCF is the inhibition of the COX enzyme, and high rates of COX inhibition have already been reported as a consequence of exposure to DCF even in low concentrations (ng L^{-1}) (Hoeger et al., 2005; Bing-Shu et al., 2017), in this study no effects of DCF were observed on *A. altiparanae* liver. Likewise, although exposure to DCF is expected to lead to downregulation of the *cox1* and *cox2* genes, Näslund et al. (2017) did not observe any effect on the expression of these genes in three-spined stickleback (*Gasterosteus aculeatus*) exposed to different concentrations of DCF (5, 20, 80 and 320 μg L^{-1}) during 28 days; while in rainbow trout (*O. mykiss*), only the lowest concentration of three evaluated (1, 10 and 100 μg L^{-1}) downregulated both genes (Cuklev et al., 2011). In humans, the mechanism of action proposed for DCF is the inhibition of the COX activity in a short period of time, considering that the maximum DCF absorption is up to 10 min, depending on the mode of administration (Davies and Anderson, 1997). Thus, it is likely that the lack of response in the present study is a consequence of the high concentration used in the bioassay (mg L^{-1}), considering that this type of compound acts in lower concentrations. Concentration-dependent physiological responses have already been observed in brown trout (*Salmo trutta*) with an inflammatory effect of DCF on the kidney at the lowest concentrations tested (0.5 and 5 μg L^{-1}), while at the highest concentration (50 μg L^{-1}), it was not possible to detect an inflammatory response (Hoeger et al., 2005).

Finally, results for both genotoxicity biomarkers showed that DCF and CAF did not trigger genetic or cytotoxic damage. Generally, the Comet assay is more sensitive to changes in the environment, being a biomarker indicated in acute exposures, whereas the micronucleus test and detection of nuclear abnormalities are indicated when the organisms are chronically submitted to genotoxic agents (Rocco et al., 2011; Braham et al., 2017). Some studies suggest that the genotoxicity of DCF depends on the sensitivity of the species, the concentration used and the time of exposure (Rocco et al., 2011; Guiloski et al., 2017). The results of the Comet assay in the present study are similar to those found by other authors who evaluated the effects of DCF and CAF in 96 h. Ghelfi et al. (2016) did not observe a genotoxic damage in blood and liver of silver catfish (*R. quelen*) exposed to 0.2, 2 and 20 μg L^{-1} of DCF for 96 h. Likewise, Santos-Silva et al. (2018) did not observe effects of CAF (0.3, 3 and 30 μg L^{-1}) on DNA fragmentation in streaked prochilod (*P. lineatus*) exposed during 24 and 168 h, which corroborates the results of the present study.

On the other hand, it is difficult to find studies that use analysis of MN and ENAs as biomarkers in acute exposure, because both are generated during mitosis, when fragments of chromosomes or whole chromosomes are not included in the nuclei of new cells during the end of the telophase (Fenech et al., 2011). Considering that the process of erythropoiesis can take an average of 12 days depending on the species (Yen-Hua et al., 2017), this biomarker would not be the most suitable to evaluate the effect to acute exposures of these compounds. The former is confirmed in the study of Rocco et al. (2012), in which zebrafish (*D. rerio*) exposed to the antibiotics erythromycin and lincomycin for different periods (7, 14, 28 and 42 days), presented a greater number of MNs with increasing exposure periods. However, in *A. altiparanae* presence of MN and a greater number of nuclear abnormalities with aluminum exposures during 24 and 96 h have already been reported (Pinheiro et al., 2019).

Analysis of the results of the present and previous studies makes it evident that physiological responses may vary when fish are exposed to DCF and CAF in concentrations in the range of ng L^{-1}, μg L^{-1} or mg L^{-1}. Exposure time is also a relevant factor, since 96 h can be too short a period to generate some responses, or on the contrary, the organisms

may have already triggered physiological responses that allowed the maintenance of homeostatic balance that were no longer observed at 96 h.

5. Conclusion

The results of the present study suggest that the mixture of compounds did not show strong synergistic or antagonistic effects. The most pronounced effects were observed with the exposure of the drugs separately. In the case of isolated effects, both compounds altered the enzymes, but only CAF caused LPO. Based on these data, the effects of CAF were more harmful in *A. altiparanae* adult males. Additionally, these pharmaceutical compounds did not damage the gills' antioxidant defenses.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This study was supported by the *Fundação de Amparo à Pesquisa do Estado de São Paulo* (FAPESP) (processes: 2017/11530-1 and 2014/16320-7), the *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq) (Process n° 152753/2017-6), and the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES) (Financing Code 001). The authors are grateful for the donation of fish by the Paraibuna Hydrobiology and Aquaculture Station, in Paraibuna city, SP. To the LAMEROA staff (IB-USP) for their assistance with collections; the Evolution and Development of Invertebrates Laboratory (Prof. Dr. Federico Brown) at the University of São Paulo (IB-USP) for using the fluorescence microscope; to the Animal Ecophysiology Laboratory (Prof. Dr. Claudia B. Martinez; UEL-Londrina/PR) for the training in genotoxicity techniques, and to IB-USP for logistics and facilities. Many thanks to Dr. Barbara Ardiles for the grammar revision of the manuscript.

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