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RESEARCH ARTICLE

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Cyto-genotoxicity and oxidative stress in common carp (*Cyprinus carpio*) exposed to a mixture of ibuprofen and diclofenac

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Abstract

Thirty million people worldwide consume each day nonsteroidal anti-inflammatory drugs (NSAIDs), a heterogeneous group of pharmaceuticals used for its analgesic, antipyretic, and anti-inflammatory properties. Recent studies report high NSAID concentrations in wastewater treatment plant effluents, in surface, ground, and drinking water, and in sediments. NSAIDs are also known to induce toxicity on aquatic organisms. However, toxicity in natural ecosystems is not usually the result of exposure to a single substance but to a mixture of toxic agents, yet only a few studies have evaluated the toxicity of mixtures. The aim of this study was to evaluate the toxicity induced by diclofenac (DCF), ibuprofen (IBP), and their mixture on a species of commercial interest, the common carp *Cyprinus carpio*. The median lethal concentration of IBP and DCF was determined, and oxidative stress was evaluated using the following biomarkers: lipid peroxidation and activity of the antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase. Cyto-genotoxicity was evaluated by micronucleus test, comet assay, and the specific activity of caspase-3. Results show that DCF, IBP, and a mixture of these pharmaceuticals induced free radical production, oxidative stress and cyto-genotoxicity in tissues of *C. carpio*. However, a greater effect was elicited by the mixture than by either pharmaceutical alone in some biomarkers evaluated, particularly in gill.

KEYWORDS

caspase-3, comet assay, *Cyprinus carpio*, micronuclei, nonsteroidal anti-inflammatory drugs, oxidative stress

1 | INTRODUCTION

In recent years, there has been growing concern about trace concentrations of pharmaceuticals in aquatic environments and their potential effects.[1,2] Some pharmaceuticals are metabolized after administration while others remain intact when being excreted. Thus, a mixture of these agents and their metabolites enters municipal sewage and wastewater treatment plants.[3] Depending on their polarity, water solubility, and persistence, some of these compounds are not completely

Abbreviations: CAT, catalase; COX, cyclooxygenase; CYP, cytochrome P450; DCF, diclofenac; GPx, glutathione peroxidase; IBP, ibuprofen; LC_{50}, median lethal concentration; LOAEL, lowest observed adverse effect level; LPX, lipid peroxidation; MDA, malondialdehyde; MEC, molar extinction coefficient; MNi, micronuclei; MS, mass spectrometer; NPX, naproxen; NSAID, nonsteroidal anti-inflammatory drug; PAR, paracetamol; pNA, *p*-nitroanilide; ROS, reactive oxygen species; SOD, superoxide dismutase; TBA, thiobarbituric acid; TCA, trichloroacetic acid; WWTP, wastewater treatment plant.

removed by treatment processes, and the unaltered pharmaceutical and/or their metabolites may re-enter surface water. These products can also enter the environment as a result of the disposal of unused or expired medications, or through pharmaceutical industry discharges.[4] Once in the environment, these compounds are considered emerging contaminants. Richardson et al.[5] define these as unregulated compounds that can pose a risk to aquatic ecosystems.

These pollutants include nonsteroidal anti-inflammatory drugs (NSAIDs), which is one of the most commonly used groups of pharmaceuticals. Takagi et al.[6] report that this group accounts for more than 70 million prescriptions annually in Britain, Spain, and Japan. They are also the sixth best-selling group of medications in the world, with an annual production of several thousand tons.[7] In Mexico, sales of 201 million dollars (USD) were reported in 2012 and an average annual growth rate of 1.3% is expected to occur from 2013 to 2017.[8] NSAIDs have diverse anti-inflammatory, analgesic, and antipyretic properties. They are a heterogeneous group and share diverse therapeutic actions and adverse effects.[9] Their mechanism of action is through inhibition of the cyclooxygenase (COX) enzymes: COX-1 and COX-2, which convert arachidonic acid to prostaglandins and thromboxanes, mediators involved in diverse homeostatic processes throughout the body.[7,9,10] The most common members of this group of pharmaceuticals in terms of consumption and biological action are naproxen (NPX), paracetamol (PAR), diclofenac (DCF), ibuprofen (IBP), and acetylsalicylic acid.[11]

Environmentally, the importance of NSAIDs lies in their inherent properties of persistence, bioaccumulative nature, water solubility, low volatility, and low tendency for adsorption by organic matter, which enable them to remain in the aquatic environment for extended periods,[12,13] favoring their uptake and bioconcentration by hydrobionts. IBP and DCF have been detected in water bodies worldwide at concentrations ranging from $\mu g\ L^{-1}$ to $ng\ L^{-1}$.[14–17] In Mexico, several studies have reported their presence in effluents and water systems. Siemens et al.[18] found IBP and DCF at concentrations of 0.12–2.30 $\mu g\ L^{-1}$ in Mexico City effluent in the Mezquital Valley; Gibson et al.[19] detected 742–4824 $ng\ L^{-1}$ in wastewater from the Tula Valley; while Felix-Cañedo et al.[20] recorded 25–100 $ng\ L^{-1}$ in surface water and 1–5 $ng\ L^{-1}$ in ground water in tributaries of the Lerma-Cutzamala system, one of the largest water supply networks in Latin America.

Trace concentrations of these compounds have been reported to induce toxicity in diverse aquatic organisms. DCF induces kidney damage and affects reproduction and growth in *Daphnia magna* and *D. longispira*.[21–23] It also induces damage on kidney, gill, and other tissues in *Salmo trutta f. fario*,[24] while IBP significantly affects the growth of several bacterial and fungal species.[25] Furthermore, previous studies have reported that NSAIDs such as DCF, IBP, NPX, and PAR induce oxidative stress and genotoxicity in *D. magna*, *Hyalella azteca*, and *Cyprinus carpio*.[26–30]

Controlled production of free radicals and maintenance of redox homeostasis are essential for the physiological health of organisms. The formation of reactive oxygen species (ROS) is induced by internal and external factors such as phagocytes, enzymes—for example, cytochrome P450 (CYP) monooxygenases—radiation, and exogenous chemical agents. Similarly, ROS production can be decreased or reversed by several enzymes, called antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx)[31]. Endogenous ROS act as a second messenger in signal transduction and are thought to be important in ion transport, host immune defenses, DNA transcription, and cellular apoptosis.[32,33] However, ROS can also elicit damage by binding covalently or irreversibly to cellular macromolecules. Oxidative stress, an imbalance between ROS production and the antioxidant defense mechanisms of a cell or tissue, elicits the irreversible oxidation of proteins, lipids, and DNA, leading to the inactivation of many enzymes and cell death. It can also affect gene expression by interfering with the activity of redox-sensitive transcription factors as well as signal transduction by oxidation of thiols.[34]

Bioindicators can be used to evaluate the toxic impact of contaminants in water bodies. Toxicity studies in fish are one of the most effective methods for understanding the deleterious effects of environmental contaminants in aquatic systems. Fish play a major role in aquatic food webs where they generally occupy an intermediate or higher position: not only are they fed upon by a variety of aquatic predators, they are also a major food source for humans around the world.[35] The common carp *Cyprinus carpio* is frequently used as a bioindicator species[36] since cyprinids are quantitatively the most important group of teleost fish cultured worldwide for commercial purposes and are also very resistant and easy to maintain.

Toxicity in natural ecosystems is not usually due to exposure to a single substance; it is the result of exposure to a mixture of several toxic agents. Thus, it is necessary to understand the impact and potential toxicity of pollutants in combination, particularly if pollution is considered to be chronic. However, very few studies have examined the effects of mixtures.[37–39] Therefore, this study aimed to evaluate the toxicity induced by sublethal concentrations of IBP and DCF in isolated form and as a mixture on diverse tissues of *C. carpio*, using oxidative stress and cytogenotoxicity biomarkers in order to assess the potential risk posed by a waterborne mixture of these pharmaceuticals to the physiology and survival of aquatic organisms. We choose DCF and IBP because they are among the most consumed NSAIDs worldwide and it has been reported that these pharmaceuticals have shown greater toxicity for aquatic organisms, plus in regard to Mexico, in 2012, was reported sales of 201 million dollars (mdd) and is expected to have a rate average annual growth of 1.3% from 2013 to 2017,[8] because of this, is very important to determine if this represents a risk for organisms.

2 | MATERIALS AND METHODS

2.1 | Test substances

IBP $C_{13}H_{18}O_2$ (CAS Number 15687-27-1, >98% purity) and DCF $C_{14}H_{11}C_{l2}NO_2$ (CAS number 15307-86-5, >99% purity), were purchased from Sigma-Aldrich (Toluca, Mexico; henceforth SIAL/T). Stock solutions were prepared by dissolving 1 g IBP or DCF in 500 mL of deionized water.

2.2 | Specimen procurement and maintenance

Three-month-old common carp (*C. carpio*) juveniles 18.4 ± 0.31 cm long and weighing 50.7 ± 7.8 g were obtained from the aquaculture facility in Tiacaque (State of Mexico), transported to the laboratory in polyethylene bags containing oxygenated water, stocked in a large tank with dechlorinated tap water (previously reconstituted with salts; see Section 2.3) and acclimated for 30 days prior to the experiment. During acclimation, carp were fed Pedregal Silver™ fish food and 3/4 of the tank water was replaced every 24 h. The physicochemical characteristics of tap water reconstituted with salts were maintained, that is, 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 (12:12 h) was maintained. During the aquaculture period, fish were not exposed to any pharmaceuticals.

All procedures were performed in accordance with the ethical protocols of care, use, and management of the species used in the testing of the Universidad Autónoma del Estado de México. The specifications mentioned in the corresponding Official Mexican Standards were also considered (NOM-062-ZOO- 1999, Technical specifications for the production, care, and use of laboratory animals).

2.3 | Median lethal concentration (LC_{50})

Test systems (120 × 80 × 40-cm glass tanks) filled with water reconstituted with the following salts: $NaHCO_3$ (174 mg L^{-1}, SIAL/T), $MgSO_4$ (120 mg L^{-1}, Sigma-Aldrich, St. Louis MO; henceforth SIAL/S), KCl (8 mg L^{-1}, Vetec, SIAL/S), and $CaSO_4{\cdot}2H_2O$ (120 mg L^{-1}, SIAL/S) were maintained at room temperature (20°C ± 2°C) with constant aeration and a natural light/dark photoperiod (12:12 h). Static systems without renewal of test solutions were used. Fish were not fed during exposure.

To determine the LC_{50} of IBP and DCF, seven exposure systems with different nominal concentrations of IBP (9.5, 18.9, 37.7, 75.2, 150.0, 300.8, and 600 mg L^{-1}) and six with DCF (9.5, 18.9, 37.7, 75.2, 300.8, and 600 mg L^{-1}) plus a NSAID-free control system were set up, and 10 randomly selected carp were placed in each. The assay was performed in triplicate, using a total of 420 fish.

Duration of exposure was 96 h, at the end of which the number of dead specimens in each system was counted. The 96-h LC_{50} of IBP and DCF and their corresponding 95% confidence limits ($P < 0.05$) were estimated by Probit analysis v3.3 (US-EPA, 2013). These data were used to estimate the test concentrations to be used in sublethal toxicity assays. The concentration-response curve in the acute toxicity assay was constructed and the lowest concentration with a statistically significant effect was assumed to be the LOAEL (lowest observed adverse effect level).

2.4 | Sublethal toxicity assays

NSAIDs were added at a nominal concentration equal to the LOAEL (i.e., 17.6 mg IBP L^{-1} and 7.10 mg DCF L^{-1}) in isolated form or as a mixture to five exposure systems with six carp each. A time dependent exposure set up was performed for the following exposure periods: 12, 24, 48, 72, and 96 h. An NSAID-free control system was set up for each period. Assays were performed in triplicate, using a total of 360 fish.

At the end of the exposure period, fish were removed from the systems and placed in a tank with 50 mg L^{-1} of clove oil as an anesthetic[40]. Anesthetized specimens were placed in a lateral position and blood was collected with a heparinized 1-mL hypodermic syringe by puncture of the caudal vessel performed laterally near the base of the caudal peduncle, at mid-height of the anal fin and ventral to the lateral line. Six hundred microliters of blood samples were collected in heparinized tubes, placed in PBS and ultrasonicated, and used for the oxidative stress determinations. Of the remaining blood 200 μL were used immediately in the micronucleus test and comet assay, and 100 μL were placed in PBS (complete to 1 mL) and stored at −70°C prior to analysis of the specific activity of caspase-3.

After puncture, specimens were sacrificed by cervical dislocation and placed in an ice bath. The gill, liver and brain were removed for evaluation of oxidative stress, placed in phosphate buffer solution (PBS) [0.138 M NaCl (SIAL/T); 0.0027 M KCl] pH 7.4 and separately homogenized. The homogenate was centrifuged at 12,500 rpm and −4°C for 15 min. Tissue samples were stored at −70°C prior to analysis. The following oxidative stress biomarkers were evaluated: lipid peroxidation (LPX) and SOD, CAT, and GPx activity. These bioassays were performed on the supernatant of the tissues or ultrasonicated blood and the assay was performed in triplicate.

2.4.1 | Determination of oxidative stress

2.4.1.1 | Determination of LPX

LPX was determined by Büege and Aust[41] method. To 100 μL of supernatant (of the tissues) or blood was added Tris-HCl buffer solution pH 7.4 (SIAL/S) until a 1 mL volume was attained. Samples were incubated at 37°C for 30 min; 2 mL TBA-TCA reagent [0.375% thiobarbituric acid (TBA, Fluka, SIAL/T) in 15% trichloroacetic acid (TCA, SIAL/S)] was added prior to shaking in a vortex. Samples were then heated to boiling for 45 min, allowed to cool, and the precipitate removed by centrifugation at 3,000 rpm for 10 min. Absorbance was read at 535 nm against a reaction blank. Malondialdehyde (MDA) content was calculated using the molar extinction coefficient (MEC) of MDA (1.56 × 10^5 M cm^{-1}). Results were expressed as mM MDA mg^{-1} protein.

2.4.1.2 | Determination of SOD activity

SOD activity was determined by Misra and Fridovich[42] method. To 40 μL of supernatant (of the tissues) or blood in a 1-cm cuvette was added 260 μL carbonate buffer solution (50 mM sodium carbonate, 0.1 mM EDTA) pH 10.2 and 200 μL adrenaline (30 mM); all reagents were obtained from SIAL/S. Absorbance was read at 480 nm after 30 s and 5 min. Enzyme activity was determined by interpolating the data on a type curve and results were expressed as IU SOD mg^{-1} protein.

2.4.1.3 | Determination of CAT activity

CAT activity was determined by Radi et al.[43] method. To 20 μL of supernatant (of the tissues) or blood was added 1 mL isolation buffer solution [0.3 M saccharose (Vetec), 1 mL EDTA, 5 mM HEPES, 5 mM

KH_2PO_4 (Vetec)] and 0.2 mL of a hydrogen peroxide solution (20 mM, Vetec); all reagents SIAL/S. Absorbance was read at 240 nm after 0 and 60 s. The absorbance value obtained for each of these times was substituted in the formula: CAT activity = $(A_0 - A_{60})$/MEC), where the MEC of H_2O_2 is 0.043 mM cm^{-1}, and results were expressed as μM H_2O_2 min^{-} mg^{-1} protein.

2.4.1.4 | Determination of GPx activity

GPx activity was determined by Gunzler and Flohe-Clairborne[44] method as modified by Stephensen et al.[45] To 100 μL of supernatant (of the tissues) or blood was added 10 μL glutathione reductase (2 U glutathione reductase, SIAL/S), 290 μL reaction buffer [50 mM K_2HPO_4 (Vetec), 50 mM KH_2PO_4 pH 7.0, 3.5 mM reduced glutathione (Fluka), 1 mM sodium azide, 0.12 mM NADPH (all SIAL/T)] and 100 μL H_2O_2. Absorbance was read at 340 nm after 0 and 60 s. Activity was estimated using the equation: GPx activity = $(A_0 - A_{60})$/MEC), where the MEC of NADPH = 6.2 mM cm^{-1}. Results were expressed as mM NADPH min^{-1} mg^{-1} protein.

2.4.1.5 | Determination of total protein content

Total protein content was determined by Bradford[46] method. To 25 μL of supernatant (of the tissues) or blood was added 75 μL deionized water and 2.5 mL Bradford's reagent [0.05 g Coommassie Blue dye, 25 mL of 96% ethanol, and 50 mL H_3PO_4 (all reagents SIAL/T), in 500 mL deionized water]. The test tubes were shaken and allowed to rest for 5 min prior to reading absorbance at 595 nm and interpolating on a bovine albumin curve (SIAL/T). Total protein content of each sample was determined and used to express the results of oxidative stress biomarkers and the specific activity of caspase-3.

2.4.2 | Evaluation of cyto-genotoxicity

2.4.2.1 | Micronucleus test

A smear of blood from each specimen was fixed in pure ethanol for 5 min, then stained with 10% Giemsa (SIAL/T) for 9 min. A total of 1000 cells from each sample were examined with a light microscope and frequency of micronuclei (MNi) was expressed as the total number of micronucleated cells per 1000 cells.[47] Criteria used to determine presence of MNi were non-linkage of small ovoid or round nuclei with main nucleus, color, and staining intensity similar to main nucleus,[48] and diameter 1/5–1/20 of main nucleus.[49]

2.4.2.2 | Comet assay

DNA damage was evaluated by comet assay as proposed by Tice et al.[50] and Lankoff et al.[51] Fully frosted slides were prepared 1 h before the sample was obtained. Slides were initially coated with a 200-μL layer of 1% agarose. Then 10 μL blood was mixed with 75 μL of 0.75% agarose. To extract DNA, slides were placed in a Coplin jar with lysis solution (2.5 M NaOH, 10 M EDTA, 10 mM Trizma base, 10% DMSO, 1% triton X-100) pH 10, for 1 h at 4°C. All reagents and gels were obtained from SIAL/S.

Slides were placed in the electrophoresis chamber for 20 min with an alkaline solution (300 mM NaOH, 1 mM EDTA) pH 13. Electrophoresis was performed at 300 μAmp, 25 V, and pH >13 for 20 min, and was stopped with a neutralization buffer (0.4 M Trizma base, pH 7.4).

The DNA was stained with 50 μL ethidium bromide (SIAL/S) and examined with a Zeiss Axiophot KS400 epifluorescence microscope equipped with a 510–560 nm filter and attached to an image analyzer with a program for measurement of the cell nucleus. A total of 100 measurements per replicate were made and the damage index (percentage of DNA damage in the tail) was obtained.

2.4.2.3 | Specific activity of caspase-3

2.4.2.3.1 | Cellular extract preparation. Jurkat cells (ATCC # TIB-152) were grown in RPMI-1640 medium containing 10% fetal bovine serumin a humidified, 5% CO2 incubator at 37°C, as recommended by ATCC. The cell density was adjusted to 106 cells mL^{-1}, and 50 ng mL^{-1} of anti-Fas mAb (clone #CH-11, MBL International, Cat. # SY-001) was added to the Jurkat cells as a positive (induced apoptosis) control. For inhibited apoptosis samples, 125 μL Z-VAD-FMK [carbobenzoxy-valyl-alanylaspartyl-(*O*-methyl)-fluoromethylketone] inhibitor (20 mM) was added to the cells at the same time as the anti-Fas mAb. Samples were incubated overnight for 16 h at 37°C in a humidified, 5% CO_2 atmosphere. Cells were harvested by centrifugation at 450×g and 4°C for 10 min. The cell pellet was maintained on ice, then washed with ice-cold PBS and resuspended in Cell Lysis Buffer at a concentration of 108 cells mL^{-1}. Cells were lysed by freeze–thaw, then incubated on ice for 15 min. Cell lysates were centrifuged at 15,000×g and 4°C for 20 min and the supernatant fraction was collected.

2.4.2.3.2 *Colorimetric assay*. A colorimetric assay kit (CaspACE™, Promega, Madison, WI), the substrate of which binds to the enzyme, releasing *p*-nitroaniline (pNA), and an UltraCruz™ microplate with flat-bottom wells were used. A reaction blank was prepared using 32 μL of caspase buffer [312.5 mM HEPES, pH 7.5; 31.25% sucrose; 0.3125% CHAPS (3- [(3-cholamido-propyl)-dimethylammonio]-1-propane-sulfonate)], 2 μL DMSO, 10 μL dithiothreitol (DTT; 100 mM), and 54 μL deionized water. The following were used: for the control and exposure groups, 32 μL caspase buffer, 2 μL DMSO, 10 μL DTT, 20 μL blood, and 54 μL deionized water; for the positive control, 32 μL caspase buffer, 2 μL DMSO, 10 μL DTT, 20 μL cellular extract, and 34 μL deionized water; for inhibited apoptosis samples, 32 μL caspase buffer, 2 μL DMSO, 10 μL DTT, 20 μL cellular extract, and 34 μL deionized water. After all solutions had been transferred, 2 μL of substrate DEVD-pNA was added to each well. The microplate was covered with parafilm and incubated for 4 h at 37°C. Absorbance was read at 405 nm and the specific activity of caspase-3 was calculated. Results were expressed as nMfree pNA h^{-1} μg $protein^{-1}$. Total protein content was determined by the Bradfordmethod.[46]

2.5 | IBP and DCF quantification by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Six carp were placed in each of five exposure systems similar to those described in Section 2.3 and a concentration equal to the LOAEL of IBP (17.6 mg L^{-1}), DCF (7.10 mg L^{-1}), or a mixture of both was added, also the NSAID-free control system used in the determinations were

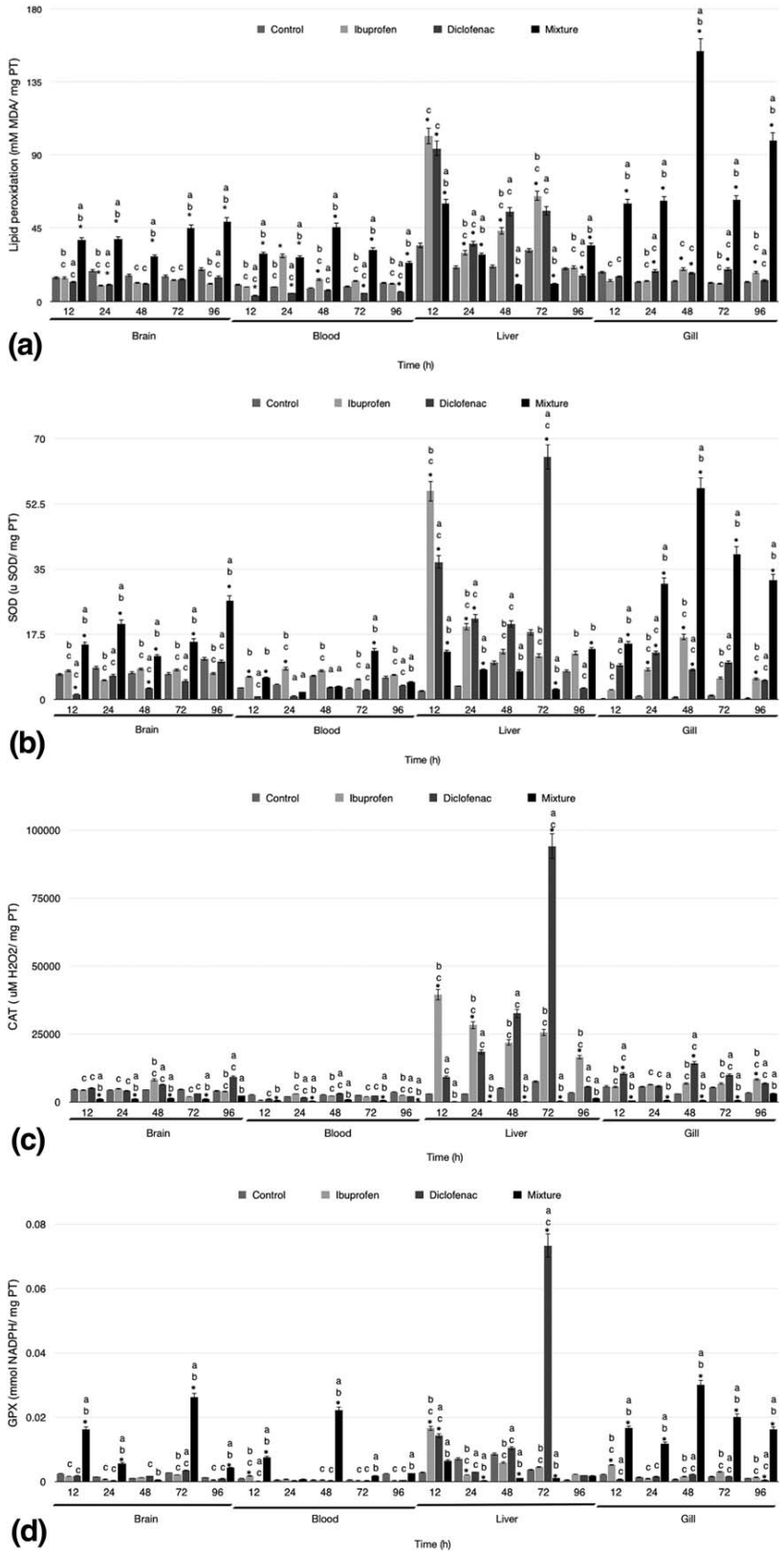

FIGURE 1 Biomarkers of oxidative stress evaluated in in brain, blood, liver, and gill of *Cyprinus carpio* exposed to ibuprofen and diclofenac in isolated form and as a mixture: (a) Lipid peroxidation, (b) Superoxide dismutase activity, (c) Catalase activity, and (d) Glutathione peroxidase activity. Values are the mean ± SE. *Significantly different from control group. Lowercase letters indicate a significant difference relative to specimens exposed to [a]ibuprofen, [b]diclofenac, and [c]the binary mixture ($P < 0.05$), ANOVA and Bonferroni

TABLE 1 Additive interaction values calculated using ibuprofen and diclofenac in isolated form, and the corresponding actual values determined with each biomarker in different tissues of *C. carpio* after exposure to a mixture of these pharmaceuticals

		Time (h)							
		24		48		72		96	
Biomarkers	Tissue	Additive interaction	Actual value	Additive interaction	Actual value	Additive interaction	Actual value	Additive interaction	Actual value
LPX (mM MDA/ mg protein)	Brain	20.057	↑38.039	22.162	↑27.554	26.439	45.156	25.582	↓10.886
	Blood	33.146	27.070	20.178	45.783	17.200	↑31.477	16.790	5.940
	Liver	65.345	↓28.634	98.208	↓10.399	120.256	10.679	37.037	↓7.588
	Gill	31.010	61.737	37.109	↑153.790	30.451	62.260	30.495	0.356
SOD activity (IU SOD/mg protein)	Brain	11.453	↑20.324	11.048	11.603	12.863	↑15.534	17.243	26.567
	Blood	9.095	1.958	10.841	3.437	7.918	13.086	10.320	↓4.633
	Liver	41.155	↓7.880	33.011	7.602	76.764	2.753	15.378	↓13.406
	Gill	20.490	31.114	24.650	↑56.657	15.648	↑39.057	10.504	↑32.005
CAT activity (mM H_2O_2/μg protein)	Brain	8973.679	1195.003	14508.806	↓1454.124	4976.454	↓1090.770	13188.581	2241.322
	Blood	4660.100	↓318.900	5352.400	↓925.600	4175.700	↓600.500	4437.600	116.600
	Liver	46700.264	219.300	54483.800	130.100	119631.100	313.700	22044.473	↓1284.330
	Gill	12120.900	↓510.700	21118.770	670.800	16536.700	535.900	15096.700	↓3119.200
GPx activity (mM NADPH/ mg protein)	Brain	0.001	0.006	0.003	↓0.001	0.006	↑0.026	0.001	0.004
	Blood	0.001	↓0.001	0.001	0.022	0.001	0.002	0.001	0.003
	Liver	0.005	↑0.000	0.016	↓0.001	0.078	↓0.001	0.004	↑0.002
	Gill	0.002	0.012	0.004	↑0.030	0.005	↑0.020	0.002	0.016
Micronucleus test (Micronuclei/ 1000 cells)	Blood	82.667	164.333	122.667	107.333	359.667	120.667	116.333	↓33.667
Comet Assay (damage index)	Blood	2.482	↓1.395	2.773	1.513	3.111	↓1.621	2.979	1.608
Caspase-3 ((nM free pNA/h)/ μg protein)	Blood	1090.641	305.338	605.161	↓306.278	331.277	140.716	790.636	277.824

LPX = lipid peroxidation, MDA = malondialdehyde, SOD = superoxide dismutase, CAT = catalase, GPx = glutathione peroxidase, pNA = *p*-nitroanilid; Additive interaction is the calculated value and result of the sum of the effect determined for the DCF and IBP by themselves, the actual value is the effect produced by the mixture DCF-IBP and experimentally determined in the study. ANOVA and Bonferroni. Up arrow and down arrow indicates a decrement or increment respect to additive interaction.

placed in order to ensure no exposure to pharmaceuticals occurred in the control group. Systems were maintained at room temperature with a natural light/dark photoperiod and provided with constant aeration. A time dependent exposure series was run for the following exposure periods: 12, 24, 48, 72, and 96 h. At the end of the exposure period, IBP and DCF were quantified in exposure system water.

IBP and DCF concentrations were determined using an Agilent 1290 Infinity HPLC unit (Santa Clara CA). The Eclipse Plus C18 RRHD (2.1 × 50 mm, 1.8 μm) chromatographic column was maintained at 40°C. The mobile phase employed was a 60:40 v/v (IBP) and a 50:50 v/v (DCF) mixture of acetonitrile and ammonium formate (10 mM, SIAL/S). Flow rate was 0.3 mL min^{-1}, run time 1.8 min and injection volume 2 μL, IBP and DCF were identified and quantified with an Agilent 6430 Triple Quadrupole mass spectrometer (MS) equipped with electrospray ionization (ESI). The ESI positive mode was used throughout. Electrospray voltage operated at 4000 V as the MS collected data in the negative ion mode. MS optimization was conducted by direct infusion of a 10 μg mL^{-1} standard solution of IBP or DCF; thereafter, the ionization mode and precursor ion mode were selected.

Five-milliliter water samples from exposure glass tanks were collected in glass sample vials and refrigerated at 4°C for subsequent measurement of test concentrations. The results are expressed as time-weighted average concentrations of IBP and DCF. Samples were acidified with 1M HCl and extraction with 5 mL (1 + 1) (v/v) hexane/ethyl acetate was conducted to extract IBP and DCF from 1-mL water samples. These samples were centrifuged at 1800×g for 10 min, and then the upper organic layer was re-extracted. Extraction was repeated until the organic layers combined and evaporated to dryness.

2.6 | Statistical analysis

In the acute toxicity assays (96-h LC_{50} of IBP and DCF), Probit analysis was performed and significance assessed by the degree of 95% LC_{50} overlap (EPA Analysis Program v3.3; US-EPA, 2013).

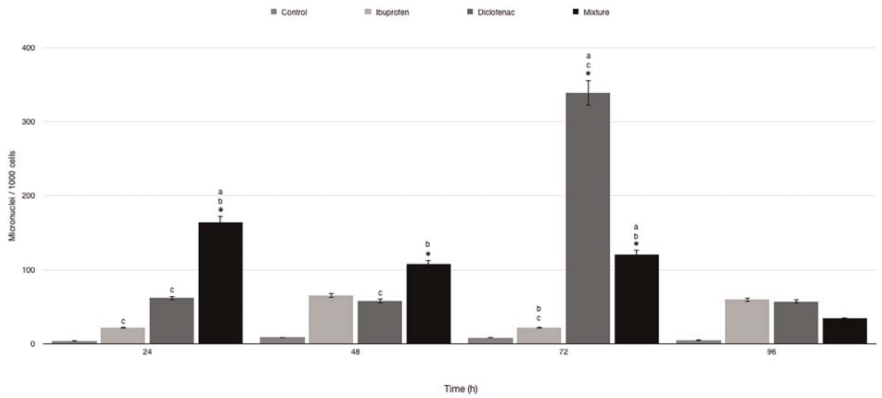

FIGURE 2 Blood levels of micronuclei in *C. carpio* exposed to ibuprofen and diclofenac in isolated form and as a mixture. Values are the mean ± SE. *Significantly different from control group. Lowercase letters indicate a significant difference relative to specimens exposed to [a]ibuprofen, [b]diclofenac, and [c]the binary mixture ($P < 0.05$), ANOVA and Bonferroni

Results of oxidative stress biomarkers, MNi, comet assay and the specific activity of caspase-3 were evaluated 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. Pearson's correlation analysis was used to examine potential correlations between IBP and DCF concentrations present in water from binary mixture exposure systems and the biomarkers evaluated in different tissues of the mixture IBP-DCF. Statistical determinations were made with SPSS v10 (SPSS, Chicago IL).

3 | RESULTS

3.1 | Determination of LC_{50}

The 96-h LC_{50} of IBP in *C. carpio* was 175.6 mg L^{-1} with a 95% confidence interval of (107.31–334.05 mg L^{-1}). The corresponding values for DCF were 70.98 mg L^{-1} and (51.66–98.14 mg L^{-1}). The χ^2 linear adjustment test was not significant at $P < 0.05$.

3.2 | Sublethal toxicity assays

3.2.1 | Evaluation of oxidative stress

3.2.1.1 | LPX

A significant increase in LPX compared to the control group ($P < 0.05$) occurred with IBP alone in blood at 24 and 48 h, in liver at 24, 48, and 72 h, and in gill at 48 and 96 h. In specimens exposed to DCF alone, LPX increased in liver at 24 h and in gill at 48 and 72 h, while in those exposed to the mixture LPX increased in liver at 24 and 96 h, and in brain and blood at all exposure times, the highest values of LPX occurring in gills of the binary mixture at all time period. A significant reduction in LPX was found with IBP in brain at 24 h; with DCF in brain at 24 h, in blood at 24, 72, and 96 h, and in liver at 96 h; and with the mixture in liver at 48 and 72 h (Figure 1).

3.2.1.2 | SOD activity

Significant increases with respect to the control group ($P < 0.05$) occurred with IBP alone in blood and liver at 24 h, and in gill at 24, 48, and 96 h; with DCF alone in liver at 24 h, and in gill at 24 and 48 h;

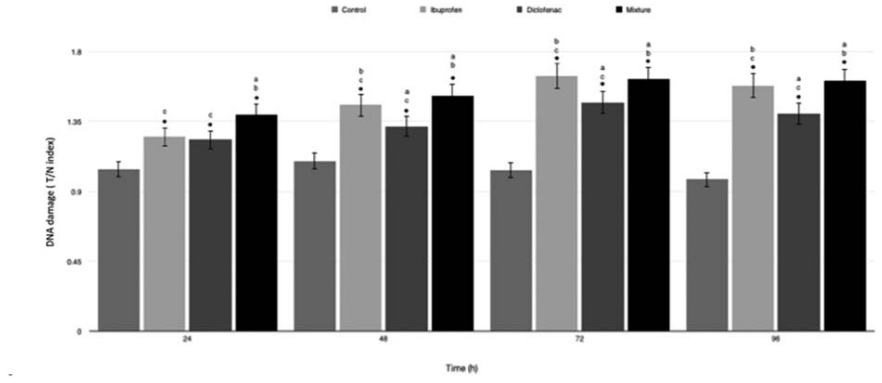

FIGURE 3 DNA damage determined by comet assay in *C. carpio* exposed to ibuprofen and diclofenac in isolated form and as a mixture. Values are the mean ± SE. *Significantly different from control group. Lowercase letters indicate a significant difference relative to specimens exposed to [a] ibuprofen, [b]diclofenac, and [c]the binary mixture ($P < 0.05$), ANOVA and Bonferroni

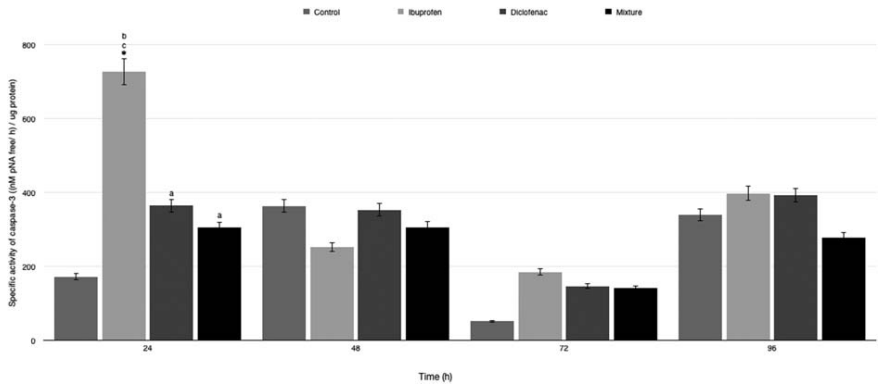

FIGURE 4 Specific activity of caspase-3 in blood of *C. carpio* exposed to ibuprofen and diclofenac in isolated form and as a mixture. Values are the mean ± SE. pNA = *p*-nitroanilide. *Significantly different from control group. Lowercase letters indicate a significant difference relative to specimens exposed to [a]ibuprofen, [b]diclofenac, and [c]the binary mixture ($P < 0.05$), ANOVA and Bonferroni

and with the binary mixture in brain and gill at all exposure times, in blood at 72 h, and in liver at 24 and 96 h. Significant reductions were observed with DCF in brain at 48 h, and with the mixture in liver at 72 h (Figure 1). The activity of the gills in the binary mixture significantly increased for all time periods

3.2.1.3 | CAT activity

Significant increases ($P < 0.05$) compared to the control group were found with exposure to IBP alone in liver at 24 and 96 h, and in gill at 96 h; and with exposure to DCF alone in liver at 72 h and in gill at 48 h; while in carp exposed to the mixture significant decreases occurred in brain at 24, 48 and 72 h, in blood at 24, 72, and 96 h, in liver at 24, 48, and 72 h, and in gill at 24, 48, and 72 h (Figure 1).

3.2.1.4 | GPx activity

Significant increases with respect to the control group ($P < 0.05$) occurred with DCF alone in liver at 72 h; and with the binary mixture in brain at 24, 72, and 96 h, and in blood at 48 h, the highest increases being observed in gill of the mixture at all exposure times. Significant reductions were found with IBP in liver at 24 h; with DCF in gill at 96 h; and with the mixture in liver at 24, 48, and 72 h (Figure 1).

Table 1 shows the additive interaction values (calculated using IBP and DCF in isolated form) and the actual values obtained with each biomarker in specimens exposed to the binary mixture. In most oxidative stress biomarkers, actual values were higher than additive interaction values at all exposure times while in biomarkers of cyto-genotoxicity a time-dependent reduction in actual values was found relative to additive interaction values.

3.2.2 | Evaluation of cyto-genotoxicity

3.2.2.1 | Micronucleus test

MNi frequency results are shown in Figure 2. Carp exposed to DCF alone evidenced a significant increase in this biomarker at 72 h ($P < 0.05$) compared to the control group, while in fish exposed to the binary mixture this increase occurred at 24, 48, and 72 h. No significant differences were found with exposure to IBP alone.

3.2.2.2 | Comet assay

A significant increase in the damage index compared to the control group ($P < 0.05$) was found at all exposure times in specimens exposed to IBP and DCF in isolated form and as a mixture. This increase was higher with IBP than DCF, while the mixture induced an even higher increase at 24, 48, and 96 h (Figure 3).

3.2.2.3 | Specific activity of caspase-3

Results of the specific activity of caspase-3 are shown in Figure 4. A significant increase with respect to the control group ($P < 0.05$)

TABLE 2 Ibuprofen and diclofenac concentrations at the different exposure times in water from single pharmaceutical and binary mixture exposure systems

Time (h)	Single pharmaceut. IBP (mg L^{-1})	Single pharmaceut. DCF (mg L^{-1})	Binary mixture IBP (mg L^{-1})	Binary mixture DCF (mg L^{-1})
0	17.56 ± 0.98	7.10 ± 0.35	17.56 ± 0.02	7.10 ± 0.05
24	13.17 ± 1.08	1.77 ± 0.13	11.39 ± 0.02*	3.55 ± 0.05*
48	12.29 ± 0.79	1.61 ± 0.06	11.01 ± 0.02*	3.31 ± 0.05*
72	11.41 ± 0.56	1.52 ± 0.06	10.64 ± 0.02	2.59 ± 0.05*
96	10.53 ± 0.91	1.39 ± 0.06	2.63 ± 0.02*	2.01 ± 0.05

Values are the mean of five replicates ± SE. IBP = ibuprofen, DCF = diclofenac. *Significantly different from single pharmaceutical exposure systems ($P < 0.05$), ANOVA, and Bonferroni.

TABLE 3 Pearson's correlation between biomarker values in the different tissues of *C. carpio* exposed to the mixture and ibuprofen and diclofenac concentrations in water from binary mixture exposure systems, at the various exposure times

Biomarkers determinated in the mixture	Tissue	IBP concentration in water from binary mixture exposure systems				DCF concentration in water from binary mixture exposure systems			
		24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
LPX	Brain	0.927	−0.693	−0.866	0.998	0.927	−0.693	−0.897	**0.998**
	Blood	0.908	−0.839	−0.956	0.908	0.908	−0.840	−0.681	0.908
	Liver	0.874	0.927	−0.680	−0.844	0.875	0.927	−0.225	−0.844
	Gill	0.936	**0.998**	0.927	**−1.000**	0.936	**0.998**	0.990	**−1.000**
SOD activity	Brain	−0.931	−0.885	−0.879	−0.853	−0.931	−0.885	**−0.999**	−0.853
	Blood	0.889	−0.815	0.880	−0.862	0.836	0.889	−0.815	**0.999**
	Liver	0.327	0.879	−0.860	−0.858	0.327	0.879	**−0.999**	−0.858
	Gill	0.921	0.985	0.985	−0.485	0.921	0.985	0.939	**1.000**
CAT Activity	Brain	0.588	−0.795	0.240	−0.857	0.588	−0.795	−0.277	−0.857
	Blood	0.777	−0.993	0.465	0.961	0.777	−0.993	−0.401	0.961
	Liver	0.601	−0.655	−0.327	0.682	0.601	−0.655	−0.756	0.682
	Gill	−0.945	0.997	0.240	**1.000**	−0.945	**1.000**	−0.277	**1.000**
GPx Activity	Brain	0.993	0.913	−0.844	**1.000**	0.993	0.913	**−0.999**	**1.000**
	Blood	0.433	−0.397	−0.997	0.577	0.433	−0.397	−0.822	0.577
	Liver	0.676	−0.572	−0.153	−0.964	0.676	−0.572	0.362	−0.096
	Gill	−0.101	0.115	0.554	−0.240	−0.101	0.115	0.896	−0.240
Micronuclei	Blood	0.322	−0.056	0.101	−0.915	0.322	−0.558	0.585	−0.915
Comet Assay	Blood	0.943	−0.983	−0.936	**0.999**	0.496	0.894	0.553	**0.999**
Caspase-3	Blood	0.885	−0.173	0.575	0.218	0.885	−0.173	−0.449	0.218

Significant correlations ($P < 0.05$) are shown in bold. A minus sign denotes a positive correlation (high values in one set correlate with high values in the other); its absence denotes a negative correlation (low values in one set correlate with high values in the other). Values near zero indicate that factors are unrelated. IBP = ibuprofen, DCF = diclofenac, LPX = lipid peroxidation, SOD = superoxide dismutase, CAT = catalase, GPx = glutathione peroxidase..

occurred only with IBP alone at 24 h; values in specimens exposed to DCF alone or to the binary mixture did not differ significantly from control group values.

3.3 | IBP and DCF quantification

Table 2 lists IBP and DCF concentrations in water from exposure systems containing these pharmaceuticals in isolated form or as a mixture. In systems with either pharmaceutical alone, NSAID concentration decreased over time. A similar behavior was observed in systems with the binary mixture; however, at 96 h, IBP concentration was markedly lower in binary mixture systems than in those with IBP alone. In the control group DCF and IBP was not observed.

Table 3 shows correlation results between biomarker values in tissues of specimens exposed to the binary mixture of IBP and DCF, with the IBP and DCF concentrations in water from binary mixture exposure systems, at the various exposure times. As can be seen, very close correlations exist between biomarker values and pharmaceutical concentrations in water from exposure systems.

4 | DISCUSSION

The 96-h LC_{50} of IBP in *C. carpio* was 175.56 mg L^{-1} in this study while the corresponding value for DCF was 70.98 mg L^{-1}. In other fish species, the LC_{50} of IBP has been reported as 173.0 mg L^{-1} in *Lepomis macrochirus*[2] and 142 mg L^{-1} in *Cirrhinus mrigala*, while the LC_{50} of DCF in juvenile *Danio rerio* ranged from 156.8 to 176.4 mg L^{-1}[52]. In our study and in others in which different species were used, DCF LC_{50} values are lower than those of IBP. Therefore, DCF can be considered to be more toxic than IBP. This difference may be due to different biotransformation metabolites; in various studies, it has shown that IBP and DCF may undergo suffer biotic and abiotic reactions. In fish, diverse P450 enzymes in the smooth endoplasmic reticulum of cells in the liver, kidneys, gills, gut, brain, heart, and gonads, among other organs, are able to biotransform xenobiotics.[53] In humans, the CYP2C9 family is known to mediate the biotransformation of IBP and DCF by hydroxylation to 2- or 3-hydroxyibuprofen and 4-hydroxydiclofenac, respectively,[54,55] while the families CYP2C8, CYP2C18, CYP2C19, and CYP2B6 mediate DCF biotransformation to 5-hydroxydiclofenac.[56] Gomez et al.[57] noted that 2-hydroxyibuprofen was the major metabolite identified

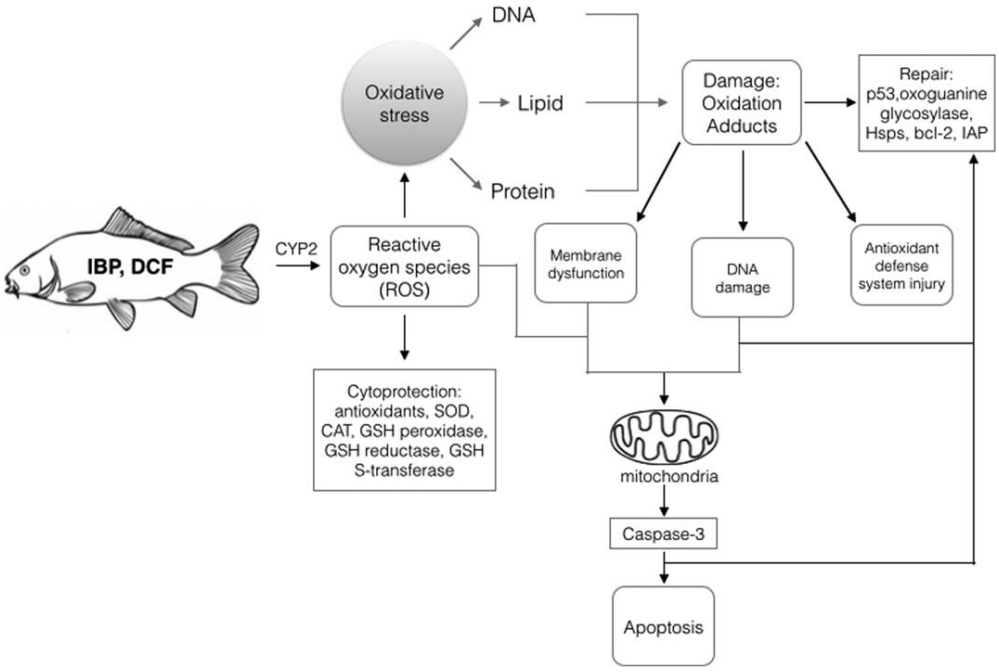

FIGURE 5 Proposed mechanisms of ibuprofen- and diclofenac-induced toxicity in *C. carpio*. CAT: catalase; DCF: diclofenac; GSH: glutathione; HSP: heat shock protein; IAP: inhibitor of apoptosis protein; IBP: ibuprofen; SOD: superoxide dismutase

in *in vitro* metabolism studies on fish, while Hoeger et al.[24] showed that DCF inhibits COX activity and therefore prostaglandin E2 synthesis in brown trout head kidney macrophages *in vitro*, thus confirming the existence in fish of the same mode of action reported previously in mammalian species. Also, IBP and DCF-derived acyl glucuronides have been shown to form covalent bonds with both intra- and extracellular proteins, with toxicological consequences.[58] In addition to biotic transformations, in water bodies, these pharmaceuticals may be photodegraded in the presence of light or biodegraded by micro-organisms to smaller, more hydrophobic and more toxic molecules. The main metabolites of IBP resulting from these processes include 4-isobutylacetophenone, 1-(6-methoxy-2-naphthyl) ethanol and 2-acetyl-6-methoxynaphthalene while those of DCF are 5,4′-dihydroxydiclofenac, 3-dihydroxydiclofenac, 4′-dihydroxymethyldiclofenac, 3′-hydroxymethyl-diclofenac, 4′-hydroxydiclofenac and 5′-hydroxydiclofenac.[59] The latter two are oxidized to benzoquinone imine intermediates, compounds that are highly toxic to aquatic organisms,[30] and therefore, the high DCF toxicity in this study could be the result this intermediates.

As can be seen in Table 2, IBP and DCF concentrations in our study decreased over time in water from single pharmaceutical exposure systems. However, in water from binary mixture exposure systems, IBP concentration decreased faster and reaches a lower concentration than in the single pharmaceutical system, while DCF concentration decreased more slowly, suggesting that these NSAIDs may have interactions in the mixture that favor the loss of IBP in the system. Furthermore, as evidenced in Table 3, these concentrations in water exposure systems can be correlated with the results obtained for different biomarkers evaluated.

A state of oxidative stress occurs when exist an excess of prooxidants that cannot be counteracted by antioxidants systems. LPX impairs biomembrane function, decreases membrane fluidity, inactivates membrane-bound enzymes and receptors, and can change selective permeability to calcium ions[60], it involves a chain of redox reactions, particularly in polyunsaturated fatty acids, which are highly sensitive to ROS-induced oxidation due to the presence of double bonds in their structure[61]. In Figure 1, the amount of MDA produced was higher in specimens exposed to the binary mixture than in those exposed to IBP or DCF alone; brain and gill being the tissues with the most LPX-induced damage. Increased LPX particularly in brain and gill was also found by Nava-Álvarez et al.[62] in *C. carpio* exposed to a mixture of DCF and acetaminophen. In phase I, biotransformation of NSAIDs, CYP produces an oxygenated intermediate—the oxycytochrome P450 complex [P450 (Fe^{3+}) O_2^-]—with subsequent release by reaction decoupling of superoxide anion, an oxidant species that damages membrane lipids,[63] besides the generation of the aforementioned reactive metabolites and intermediates, on the other hand, since NSAIDs affect the mitochondrion and therefore oxidative phosphorylation, increased ROS production may occur, particularly of $O_{2-}^{\cdot}$, and as a result, an increase in LPX, and these processes may be explained the increase found. To mitigate the negative effects of ROS, fish—like other vertebrates—possess an antioxidant defense system. Antioxidant defenses are induced by environmental contaminants under prooxidant conditions. The main enzymatic antioxidant is SOD, these include the manganese-complexed enzyme in mitochondria (MnSOD) and the copper/zinc-complexed enzyme (CuZnSOD) present in the cytosol and extracellular surfaces.[64] SOD catalyzes the conversion of superoxide anion (O_2) to hydrogen peroxide (H_2O_2),[65] which is metabolized to O_2

and water by CAT, associated primarily with peroxisomes and detoxification,[66] and GPx.[35] In our study, the binary mixture of IBP and DCF induced a higher increase in SOD activity than either pharmaceutical alone (Figure 1). It is therefore inferred that, particularly in brain and gill, SOD counteracts LPX-induced damage. The increased SOD activity found may be explained by the fact that both IBP and DCF oxidative metabolism favor formation of the anion radical O^{2*}.[63] Similar results were obtained in the freshwater bivalve *Dreissena polymorpha* exposed to a mixture of DCF, IBP, and PAR. In our study (Figure 1), CAT activity decreased in groups exposed to the mixture, the lowest values being recorded in liver. This decrease may be due to overproduction of H_2O_2. GPx is a cytosolic enzyme and the most important peroxidase that reacts with H_2O_2, reducing it to H_2O and alcohol using GSH as a reducing agent.[67,68] In this study, GPx activity was highest in gill and brain (Figure 1), as was also the case with SOD activity (Figure 1), possibly showing that these enzymes act at the cytosol of cells reducing H_2O_2 formed from the reaction of SOD. Bagnyukova et al.[69] report that LPX products are apparently involved in the regulation of antioxidant enzymes. Thus, increased ROS production in this study may also explain the increased SOD, CAT, and GPx activity observed.

Monitoring of clastogenic effects of pollutants is of primary interest in aquatic environmental mutagenesis for determining the pollution-related stress in living organisms.[70] MNi test with fish has been shown to be a useful in vivo technique for genotoxicity testing, MNi are chromosome fragments or whole chromosomes that are not included in the main nucleus, and are seen as small nuclei in the cytoplasm of cells in interphase.[71] MNi arise in peripheral blood erythrocytes as a result of DNA damage to cells mainly in the S-stage of interphase. In our study, the binary mixture induced significant increases in MNi at 24, 48, and 72 h, although peak values were recorded with DCF alone at 72 h (Figure 2). This behavior—i.e., MNi values increasing during early exposure times and decreasing thereafter—has been observed in other species exposed to different genotoxic agents. A decrease in MNi numbers after a phase of increase depends on the type of genotoxic concentration used, manner of administration, and genetic response in each species.[47,72,73] The increase in MNi found in this study may be due to breakage of DNA and/or chromosome mis-segregation, events resulting from aneugenic and clastogenic effects elicited by ROS and some xenobiotics, including DCF and IBP.[74] ROS production has been shown to induce single-strand breaks and base modifications,[75] which may explain the increased MNi frequency and damage index results found in the present study. On the other hand, the decrease observed at 96 h in MNi could be due to an increased antioxidant activity, mainly of SOD and GPx activity, of the organisms in response to oxidant damage, which is evident in Figure 1. Comet assay results in this study show that significant damage was induced in all exposed groups at all exposure times, a higher rate of damage being induced by the mixture (Figure 3). In the DNA molecule, the nucleophilic groups of deoxyribose and nitrogenous bases are exposed to electrophilic attack by ROS which reach the cell nucleus and are formed as a result of external agents or cellular metabolic processes.[76] Different types of oxidative DNA damage have been reported, including single or double-strand breaks in the sugar-phosphate backbone, modification of nitrogenous bases (thymine ring saturation and fragmentation) and formation of DNA–protein or DNA–DNA crosslinks through diverse mechanisms: modification of DNA bases—OH radical action leads to over 20 modifications, the most common being 8-hydroxy-2′-deoxyguanosine (8-OHdG) which has high mutagenic potential like 5-hydroxymethyl-2′-deoxyuridine—depurination of DNA bases (apurinic or apyrimidinic sites formed by cleavage of the glycosidic bond, which may result from OH· radical attack on sugar), and strand breaks (due to cleavage of the phosphodiester bond, occurring frequently by free radical attack on deoxyribose of the DNA backbone).[77]

Cytotoxicity is a change in basic cell functions leading to damage that can be detected. Apoptosis or programed cell death is an ATP-dependent active process, characterized by breaking of the cell into apoptotic bodies, nuclear condensation, and caspase activation, which does not affect neighboring cells. There are two main routes to apoptotic death: the extrinsic pathway, initiated by the binding of specific cell membrane receptors and resulting in activation of caspase-8 which begins the cascade activation of other molecules leading to cell death; and the intrinsic pathway, initiated by rupture of the mitochondrial membrane and formation of pores through which caspase-9 activating factors (cytochrome c) are released, initiating the cascade leading to death by apoptosis; it should be noted that both pathways in turn lead to the activation of the main effector caspase, caspase 3, which degrades other protein substrates into the cells to trigger apoptotic processes.[78] In our study (Figure 4), IBP alone at 24 h was the only factor inducing a significant increase in the specific activity of caspase 3. Since COX inhibition is the mechanism of action of NSAIDs, it has been suggested that decreased cellular levels of prostaglandin E2 (PGE2) and increased levels of arachidonic acid may be involved in inhibition of cell proliferation and induction of apoptosis.[79] An increase in the cellular concentration of arachidonic acid can alter mitochondrial membrane permeability and elicit cytochrome c release, leading to apoptosis.[80,81] Arachidonic acid also increases the production of ceramide, a potent apoptosis inducer.[82] Diverse studies suggest that increased ROS formation, a deficit in antioxidant defenses, decreased DNA repair mechanism efficiency, proteolysis and loss of immune system regulation contribute to increased oxidative stress.[83] Also, protein changes such as carbonylation, nitration, and protein–protein crosslinking are generally related to loss of function and can lead on the one hand to splitting and degradation of damaged proteins and on the other to formation of aggregates resulting in protein accumulation, cytoplasmic inclusions, and eventual cell death[84]. Any stressful stimulus (exposure to ROS, DNA damage, or increased extracellular calcium induced by prostaglandin inhibition) can initiate the intrinsic pathway of apoptosis, inducing changes in mitochondrial membrane permeability and edema leading to reduced membrane potential and release of cytochrome c, which binds to Apaf-1, which in turn uses ATP to activate caspase 9. The latter activates the effector caspase 3, which hydrolyzes specific substrates leading eventually to cell death. All of these, may explain the increase observed in the specific activity of caspase 3 during early

exposure times in our study; while the decrease may be due to the fact that the cell also possesses anti-apoptotic proteins (Bcl-2), inhibitor of apoptosis proteins (IAPs) and heat shock proteins (HSPs), which act at different levels of the apoptosis cascade preventing its activation; addition to antioxidant enzymes that prevent oxidative damage in cells whose increase can also be seen at different exposure times.

Based on the results observed in this study, we can assume that the IBP and DCF in contact with *C. carpio*, are biotransformed, especially by the family of cytochrome CYP2, generating ROS, which if not counteracted by different cellular mechanisms of antioxidant protection, can generate a state of oxidative stress, which may result in oxidative damage and adducts at DNA, lipid, and protein level, causing dysfunction in cell membranes, DNA damage, and antioxidant defense system lesions. If damage to the membrane or the DNA is severe, it can activate the intrinsic pathway in the mitochondria which subsequently initiates the cascade that leads to the activation of the main effector caspase, caspase 3, and cell death by apoptosis, which can be inhibited, or possibly repaired by different cellular mechanisms (Figure 5).

5 | CONCLUSIONS

Our results show that may be a correlation between the loss of NSAID concentrations in water systems and biomarkers evaluated over exposure time. It is therefore proposed that DCF and IBP both in isolated form and as a mixture induce free radicals, oxidative stress, and cyto-genotoxicity in tissues of *C. carpio*. Induced effects are greater with the binary mixture than with either pharmaceutical alone, particularly in gill, and it is possible to infer that potentiation interactions take place between DCF and IBP, while antagonistic interactions occur in biomarkers of cyto-genotoxicity This study provides evidence of oxidative stress and cyto-genotoxicity as a result of sublethal exposure to NSAIDs in the common carp.

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