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Low concentrations of ciprofloxacin alone and in combination with paracetamol induce oxidative stress, upregulation of apoptotic-related genes, histological alterations in the liver, and genotoxicity in *Danio rerio*

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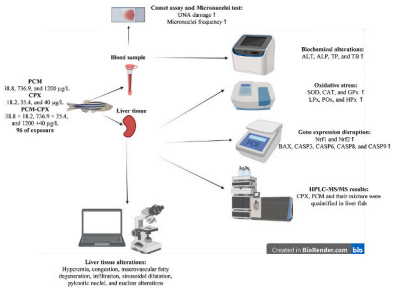
HIGHLIGHTS

- The liver function of fish was affected after 96 h of exposure to ciprofloxacin.
- Apoptosis- and oxidative stress gene-related were upregulated by ciprofloxacin.
- Ciprofloxacin induced a significant oxidative stress response in the liver fish.
- By the comet assay and micronuclei test, we found that ciprofloxacin induced genotoxicity.
- Mixtures produced more damage in all biomarkers compared to ciprofloxacin alone.

GRAPHICAL ABSTRACT



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ABSTRACT

Nowadays, there are countless articles about the harmful effects of paracetamol (PCM) in non-target organisms. Nonetheless, information regarding the toxicity of ciprofloxacin (CPX) and the CPX-PCM mixture is still limited. Herein, we aimed to evaluate the hepatotoxic and genotoxic effects that ciprofloxacin alone and in combination with paracetamol may induce in *Danio rerio* adults. For this purpose, we exposed several *D. rerio* adults to three environmentally relevant concentrations of PCM (0.125, 0.250, and 0.500 μg/L), CPX (0.250, 0.500, and 1 μg/L), and their mixture (0.125 + 0.250, 0.250 + 0.500, and 0.500 + 1 μg/L) for 96 h. The blood samples showed CPX alone and in combination with PCM damaged the liver function of fish by increasing the serum levels of liver enzymes alanine aminotransferase and alkaline phosphatase. Moreover, our histopathological study demonstrated liver of fish suffered several tissue alterations, such as congestion, hyperemia, infiltration, sinusoidal dilatation, macrovascular fatty degeneration, and pyknotic nuclei after exposure to CPX alone and in combination with PCM. Concerning oxidative stress biomarkers and the expression of genes, we demonstrated that CPX

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and its mixture, with PCM, increased the levels of antioxidant enzymes and oxidative damage biomarkers and altered the expression of Nrf1, Nrf2, BAX, and CASP3, 6, 8, and 9 in the liver of fish. Last but not least, we demonstrated CPX alone and with PCM induced DNA damage via comet assay and increased the frequency of micronuclei in a concentration-dependent manner in fish. Overall, our results let us point out CPX, even at low concentrations, induces hepatotoxic effects in fish and that its combination with PCM has a negative synergic effect in the liver of this organism.

1. Introduction

Ciprofloxacin (CPX) is an antibiotic of the fluoroquinolone class used to treat bacterial infections such as urinary tract infections, typhoid fever, gastrointestinal infections, lower respiratory tract infections, and anthrax (Thai et al., 2020). It is one of the most commonly prescribed antibiotics in the US, with an annual number of prescriptions of 173 per 1000 beneficiaries (Durkin et al., 2018). Moreover, in Europe, this drug account for 48.9% of consumption in the community expressed in DDD per 1000 inhabitants per day (Adriaenssens et al., 2021). Paracetamol (PCM) is a non-anti-inflammatory drug with analgesic properties used for the relief of muscle and joint pain, cold and flu symptoms, common headaches, and fever (McCrae et al., 2018). Moreover, as it is less toxic than aspirin and does not produce anemia, some studies have suggested its usage as an anti-platelet agent (Ogemdi, 2019). In 2019, PCM summed a total of 740 million USD in sales (Acetaminophen Global Market Report, 2018); however, recent trends have shown that its consumption has increased through the years. Thus, it is forecasted that the sales of this drug will rise to 780 million USD by 2023 (Acetaminophen Global Market Report, 2018) (see Fig. 8).

As PCM and CPX are among the most consumed drugs in the world and are not easy to remove from wastewater treatment plants (Lee et al., 2020; Lu et al., 2020), both drugs are released every day into the aquatic environment. Up to date, PCM has been reported in wastewater treatment plants (WWTPs) effluents at concentrations of 1.7 μg/L to 3000 μg/L and in surface waters at concentrations of 13.21 μg/L to 30.4 μg/L (Okuda et al., 2008; Spongberg et al., 2011; Campanha et al., 2015; SanJuan-Reyes et al., 2015). Moreover, CPX has been found as well in WWTPs effluents at concentrations of 1.3 μg/L to 341 μg/L and in surface waters at concentrations of 0.25 μg/L to 5528 μg/L (Andrieu et al., 2015; He et al., 2015; Riaz et al., 2017; Gothwal and Shashidhar, 2017). Therefore, high concentrations of both drugs have been found in worldwide water bodies, posing a real threat to aquatic species.

Nowadays, there are a lot of articles talking about the toxic effects that PCM may induce in several aquatic species (Gómez-Oliván et al., 2012; Antunes, et al., 2013; Nunes et al., 2015, 2017; Daniel et al., 2019; Liu et al., 2019; Perussolo et al., 2019; Nogueira et al., 2019). However, the data concerning CPX toxicity in non-target organisms has been scarcely researched. Up to date, eight studies that we are aware of have studied the toxic effects of CPX in fish. The first of these was performed by Martins et al. (2012), who indicated that chronic exposure to CPX (1.79–25.5 mg/L) negatively affected the size of neonates from the first brood. Analogously, Wang et al., 2014 and Yin et al. (2014) showed that concomitant administration of CPX (>37.5 mg/L) with ofloxacin, norfloxacin, and enrofloxacin affected the normal formation of *D. rerio* larvae. In agreement with these results, Rosas-Ramírez et al., 2021 demonstrated CPX (5–40 μg/L) reduced the survival rate of *D. rerio* embryos due to the high prevalence of structural malformations it induced on them. Moreover, the authors indicated that CPX induced oxidative damage in *D. rerio* larvae by producing lipid peroxidation. Nonetheless, unlike the above studies, Plhalova et al. (2014) and Nogueira et al. (2019) pointed out CPX at concentrations that range from 0.7 μg/L to 3 000 μg/L and from 0.005 μg/L to 0.488 μg/L, respectively, reduced the levels of TBARS and did not affect the growth or cause developmental changes in *D. rerio*. Besides the embryotoxic effects above-described, Shen et al. (2019) showed that the expression of calcium channel genes, cacna1a, and cacna1b, was promoted, and gene expression of cardiac troponin C, tnnc1a, and ATPase, atp2a1l, was inhibited after *D. rerio* larvae exposure to CPX (156–1949 mg/L). Moreover, they denoted that though CPX did not generate morphological abnormalities in the heart, it disrupted the normal function of the cardiovascular system. Similarly, in another study performed by Ramesh et al. (2021), the authors pointed out the levels of LPX, SOD, and GST increased in the gills, kidneys, and liver of *Cirrhinus mrigala* after treatment with CPX (1 and 1.5 μg/L). Furthermore, these concentrations of CPX also caused significant changes in sodium, potassium, and chloride levels in the plasma and some tissue alterations changes in gills, liver, and kidneys of fish.

Concomitant administration of more than one drug is a common practice in medical science (Ahsan et al., 2011). Nonetheless, drug-drug interactions can modify the drugs by forming chemical complexes, increasing the effect, and inducing or inhibiting the hepatic metabolism and elimination rate (Ahsan et al., 2011a,b). A clear example of these drug-drug interactions occurs between CPX and paracetamol (PCM) (Issa and El-Abadla, 2006; Issa et al., 2007; Ahsan et al., 2011a,b; Ahsan et al., 2011). Issa and El-Abadla (2006), for instance, demonstrated that concomitant administration of CPX with PCM resulted in prolonged half-life time of the latter in ten healthy female volunteers. Moreover, Issa et al., 2007, indicated that the concentration-time profile of CPX increased in ten healthy male volunteers when they administered volunteers the CPX-PCM mixture. Thus, a pharmacokinetic interaction may have occurred both in female and male volunteers.

Though previous studies have shown CPX may interact with PCM, changing the time they remain in the organism and their bioavailability, only one study that we are aware of has studied the toxic effects of this mixture. Rosas-Ramírez et al., 2021 found CPX-PCM mixture (5 μg/L of CPX and 150 μg/L of PCM; 18.2 μg/L of CPX and 438.8 μg/L of PCM; 35.4 μg/L of CPX and 736.98 μg/L of PCM; 40 μg/L of CPX and 1200 μg/L of PCM) produced oxidative stress and structural malformations in *D. rerio* embryos after 96 h of exposure. Moreover, they indicated that the mixture CPX-PCM produced more severe effects in embryos than drugs alone. Thus, this data suggest CPX-PCM mixture has a negative synergic effect in organisms.

Since few studies have assessed the toxic effects of CPX and the CPX-PCM mixture, we aimed to evaluate the hepatotoxic effects and genotoxic effects that environmentally relevant concentrations of this drug alone and in combination with PCM may induce in *D. rerio* adults. We hypothesize that CPX will induce hepatotoxic effects in fish and that mixture CPX-PCM will have a more negative impact on organisms than drugs alone.

2. Method

2.1. Chemical compounds

We purchased CPX (CAS number: 85 721-33-1) and PCM (CAS number: 103–90-2) from Sigma-Aldrich (St. Louis, MO). Moreover, all other compounds mentioned in the methodology were acquired from Sigma-Aldrich as well.

2.2. Zebrafish housing

Wild-type *D. rerio* adults (AB strain) were housed in aquaria of 100 L of capacity provided with UV-sterilized and charcoal-filtered water.

Temperature (27 ± 1 °C), light-dark cycles (14 h light: 10 h dark), and water quality parameters (Table 1) were examined and kept constant in all aquaria during the zebrafish housing and exposure. Fish were fed four times a day with Spirulina flakes (Ocean Nutrition, US).

2.3. Zebrafish exposure

800 *D. rerio* adults were allocated to 10 different aquaria (80 fish per aquaria) of 80 L of capacity. Nine of the ten aquaria were assigned one of the three tested concentrations of PCM (0.125 μg/L, 0.250 μg/L, and 0.500 μg/L), CPX (0.250 μg/L, 0.500 μg/L, and 1.0 μg/L) or the binary mixtures (M1, 0.125 μg/L of PCM and 0.250 μg/L of CPX; M2, 0.250 μg/L of PCM and 0.500 μg/L of CPX; M3, 0.500 μg/L of PCM and 1.0 μg/L of CPX). Moreover, we assigned an aquarium as the control group. All along the 96 h of exposure, we renewed water from all aquaria every other day, being careful to keep the nominal concentrations constant.

2.4. Biochemical parameters

After exposure to CPX, PCM, and the binary mixtures, we anesthetized fish with MS-222 (0.1%) to subsequently collect their blood. Blood samples were collected using the established method by Babaei et al. (2013). Briefly, by using a pair of scissors, we cut the caudal fin of an anesthetized fish, which was then put, with the wound pointing down, into a perforated tube that had been previously placed into another 1.5 mL microcentrifuge tube. This assembly was then centrifuged at 40 g for 5 min at 11 °C. Subsequently, another cut was done closely behind the existing wound to remove the clot formed. We centrifuged fish once again as above, and the blood collected was used for the evaluation of biochemical parameters and genotoxicity. To evaluate the biochemical parameters, we used a veterinary automated chemistry analyzer (Seamaty SMT-120 VP).

2.5. Oxidative stress evaluation

Once the blood was collected, we euthanized fish using the hypothermic shock method (2 °C–4 °C) and extracted and gathered their livers in Eppendorf tubes containing 1 mL of phosphate buffer solution at a pH of 7.4. From each of the treatments of CPX, PCM, and binary mixtures, we used 20 livers to evaluate the oxidative stress biomarkers. To evaluate these biomarkers, we opted to use different spectrophotometry and colorimetric methods, see Table 2. For this effect, we treated the samples as Elizalde-Velázquez et al. (2021a, 2021b) described. Briefly, by using a rotor-stator homogenizer, livers were homogenized for 20 s. Next, we split up the samples into two Eppendorf tubes. Thus, one of the tubes contained 300 μL of the homogenate and 300 μL of trichloroacetic acid (20%), while the other one only contained 700 μL of the homogenate. Finally, we centrifuge the tubes at 11 495 rpm and 12 500 rpm for 15 min (4 °C), respectively, using a refrigerated microcentrifuge, and then used the supernatant to determine oxidative stress response (see Table 3).

2.6. Histopathological damage evaluation

The livers from 20 fish per treatment group were individually and horizontally put in tissue cassettes (SIMPORT Histosette) and then fixed by using a buffered formalin at 10%. After 15 days of fixation, we decalcified the liver by using a Davidson solution (Miki et al., 2018) and then submitted it to slow dehydration and infiltration in an automated tissue processor (LEICA TP1020). Once samples were dehydrated and infiltrated, we immersed them in paraffin and frozen them with the help of a modular tissue embedding center (LEICA EG 1140H and LEICA EG 1140C). By using a rotatory microtome (MICROM HM 315), we then sectioned samples to a thickness of 5 μm. Histological sections were collected in a water bath at 37 °C and placed on clean glass slides. Finally, we dyed all the slides containing the sections of fish livers with hematoxylin (30 min) and eosin (10 min) and covered them with a solution of xylol and resin.

For the histopathological analysis, we observed the samples in an optical microscope (ZEISS Primo Star) coupled to a digital camera (CANON Powershot G10) and a laptop (TOSHIBA Satellite). Photomicrographs were taken by using the software CANON Utilities Remote Capture DC 3.1.0.5 and edited with the help of the software ZEISS AxiS 40 V 4.8.0.0. We characterized histopathological alterations according to the method of Bernet et al. (1999). Once alterations we characterized, we established their presence or absence in the liver and then calculated the prevalence of the injuries in each treatment group. To calculate the prevalence of the injuries, we split the number of alterations that the liver presented between the total of alterations, and then we multiply this value by 100 (Ramírez-Trejo et al., 2019).

The histopathological index was established by clustering the alterations into three reaction patterns. The reaction patterns for the liver were circulatory disturbances, inflammation, and progressive changes. We assigned each alteration with a pathological importance factor (w) and a score value (a) (Bernet et al., 1999; Matus et al., 2018). The pathological importance factor represents the level of damage in an organ after any alteration and ranges from 0 to 3. Thus, alterations such as hyperemia, intracellular edema, congestion, macrovascular fatty

Table 1
Water quality parameters.

Parameters	Value
Un-ionized ammonia	0.008 ± 0.004 mg/L
Nitrite (NO_2^-)	0.024 ± 0.006 mg/L
Nitrate (NO_3^-)	2.3 ± 0.5 mg/L
pH	7.28 ± 0.12
Dissolved Oxygen	8.8 ± 0.5 mg/L

Table 2
Methods used for the oxidative stress biomarkers determination.

	Biomarker	Method used
Tube 1	LPx	Buege and Aust (1978)
	HPx	Jiang et al. (1992)
	POx	Levine, et al. (1994)
Tube 2	SOD	Misra and Fridovich (1972)
	GPx	Günzler and Flohé (1985)
	CAT	Radi et al. (1991)

Table 3
Primers used in qRT-PCR.

Gene	Accession number	Forward primer	Reverse primer	Reference
Nrf1	NP_998020	TTT GGT TCC CGA TGA AGA CG	TGA TTA GCG TGA GAC TGA GC	Sant et al. (2017)
Nrf2	NP_878309	ACC CAA TAG ATC TAC AGA GC	GGT GTT TGG ACA TCA TCT CG	Sant et al. (2017)
BAX	AF231015	GGC TAT TTC AAC CAG GGT TCC	TGC GAA TCA CCA ATG CTG T	Soares et al. (2017)
CASP3	NM181601.5	CCG CTG CCC ATC ACT A	ATC CTT TCA CGA CCA TCT	Félix et al. (2018)
CASP 6	NM001020497.1	AGG ACA GCG CTT CAG CAG GAC A	TGA GAG CCA TTC CCC GTC TCT TGT	Félix et al. (2018)
CASP 8	NM_131510	GTT TTG GGC ACA GAT GGT AA	TAC TGT GGC CAT TCC GAT CA	Jiang et al. (2014)
CASP 9	NM_001007404	CGG AGG AGG TGA GAA GGA TAT	TCC AGC ACA CGA TCA AGA TT	Jiang et al. (2014)

degeneration, and sinusoidal dilatation have a score of 1, as these are of minimal pathologic importance. Moreover, alterations such as infiltration, nuclear alterations, pyknotic nuclei formation, and necrosis have a score between 2 and 3, as these are moderate to severe injuries, leading to partial to total loss of liver function. The score value represents the occurrence of the alterations and ranges from 0 to 6. A value of 0 indicates that alterations did not occur, whereas a value of 6 means alterations frequently occurred. The histopathological index (HI) was calculated with the following formula $HI = \Sigma (w*a)/\Sigma M$, where M represents the maximum attributable value for an alteration.

2.7. qRT-PCR

RNA was isolated from a pool of 20 livers by using a RNeasy® kit of Qiagen. After isolation, RNA concentrations were determined through the 260/280 ratio using a spectrophotometer (THERMO Scientific NanoDrop, 2000/2000c). Moreover, samples purities were assessed by using agarose (1%) gel electrophoresis. We performed the reverse transcription reactions using 1.0 μg of the total RNA and the QuantiTect® Reverse Transcription Kit (QIAGEN, Hilden, Germany, REF, 205313). Reaction conditions were as follows: 42 °C for 15 min and 95 °C for 3 min cDNA was used as a template for qRT-PCR. Genes tested were involved in different biological pathways connected with the toxicity of PCM and CPX. qRT-PCR was performed using a Rotor-Gene Q (Qiagen). We performed each reaction in a 50 μL solution containing 0.3 μmol primers, 25 μL 2X SYBER Green QuantiTec® (QIAGEN, Hilden, Germany), and 500 ng of cDNA template. Reaction conditions were as follows: 94 °C for 15s, followed by 35 cycles of 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. B-actin was used as the housekeeping gene to normalize all the samples.

2.8. Comet assay

To evaluate the genetic damage induced by CPX alone and with PCM, we performed the unicellular gel test, following the protocols established by Tice et al. (2000). Briefly, one day before the test, we placed agarose (1%) in the slides until we formed a thin layer on them. The test day, we mixed 10 μL of each lymphocyte suspension with 75 μL of agarose (0.75%) and then placed 75 μL of the mixture on the previously prepared slides. For lymphocytes obtainment, we centrifuged 300 μL of blood plasma from fish exposed to each treatment and then resuspended them in mounting solution. Once we placed lymphocytes on slides, we embedded the slides in the lysis solution, which consisted of 2.5 M sodium chloride, 10 mM Trizma, 10% DMSO, 1% Triton and pH 10, for an hour at 4 °C. Next, we positioned the slides in the electrophoresis chamber for 20 min under the following conditions: 900 mL cold alkaline solution (300 mM sodium hydroxide and 1 mM EDTA) at pH 13; 300 mA; and 25 V. To stop the process, we used a neutralization buffer (0.4 M of Trizma base) at a pH of 7.4. Finally, we left the slides to dry and stain them with 50 μL of ethidium bromide. We examined the dyed slides with a fluorescence microscope (Zeiss) by using the program Image-pro plus 5.0 (Media Cybernetics) and a wave filter of 450–490 nm. 100 measurements were done per treatment and the damage was obtained employing the DI method that is the ratio of the length of the tail of the comet and the nuclear diameter.

2.9. Micronuclei test

For the micronuclei test, we used the method reported by Çavas and Ergene-Gözükara, 2005 and analyzed 1 000 blood cells. Briefly, we place a drop of blood from fish exposed to each treatment on a slide, so we can then do a smear. Next, we dyed smears with 10% Giemsa stain for 9 min and observed the micronuclei as DNA fragments in the erythrocytes under an optical microscope.

2.10. Determination of PCM, CPX and their mixture

For drugs determination in the liver, we extracted CPX and PCM from fortified liver fish through a simple liquid extraction procedure. Briefly, we homogenized fortified samples as above and then added a volume of 0.125 mL of formic acid (1%), 0.25 mL of methanol, and 0.25 mL of acetonitrile to these. Next, the glasses tubes with the samples were subjected to a vortex and a vertical shaker for 10 min to be then centrifugated for 12 min at 10 000 rpm. We repeated this process and collected the supernatant from each extraction into another glass tube. After extraction, we evaporated the extraction solvent to dryness with an Eppendorf concentrator plus at 45 °C. We then reconstituted the powder got from the vacuum with 1 mL of *n*-hexane and 0.5 mL of the mobile phase. This mixture was vortexed, defatted, and centrifugated (10 000 rpm for 5 min). Finally, we filtered the bottom layer through a 0.22 μm nylon membrane filter, and subsequently, we injected 10 μL of the filtered liquid under optimized conditions. Concerning drug determination in water, we took 10 mL of water from each of the systems after 96 h of exposure.

To analyze the above samples, we used an Agilent 1260 HPLC system coupled to an API 5500 Qtrap MS with a Turbo V ion spray source. The source parameters were maintained as follows: voltage 5.5 kV; collision gas: medium; temperature 400 °C; desolvation gas flow 500 L/h. Chromatographic separation was carried out on a reversed-phase C18 column (50 mm × 3.0 mm, particle size 2.7 μm). The mobile phase was composed of formic acid in water (0.1%): formic acid in methanol (0.1%) (20:80). We eluted the samples at a flow rate of 400 μL/min. For data acquisition and data processing, we used the Analyst 1.6 software. We prepared working standards of PCM (0 μg/L – 1.0 μg/L) and CPX (0 μg/L – 1.5 μg/L) by diluting the stock solution in drug-free liver tissue homogenates. The accuracy of the proposed process was set by the control spiking method, which we carried out by spiking drug-free liver tissue homogenates with PCM and CPX at three singular percentages (80%, 100%, and 120%).

2.11. Ethical approval

All procedures performed in this study were in fulfillment of the ethical standards of The Ethics and Research Committee of the Autonomous University of the State of Mexico (approval ID: RP. UAEM. ERC.132.2020).

2.12. Statistical analysis

We assessed all the data through a One Way ANOVA followed by a post hoc test (Student Newman Keuls) and expressed them as the mean ± standard deviation (SD). Sigma Plot 12.3 software was used for statistical analysis. A Principal Component Analysis between variables and treatment groups was carried out using the R software. We considered a significance of $p < 0.05$.

3. Results

3.1. Biochemical parameters

ALT, ALP, total bilirubin, and total protein values are shown in Table 4. As can be seen in this table, all biochemical parameters are above the reference value and are significantly different from the control group (ALT: $F(9,29) = 155.191$; $p < 0.001$; n = 3; ALP: $F(9,29) = 42.147$; $p < 0.001$; n = 3; Total Bilirubin: $F(9,29) = 18.964$; $p < 0.001$; n = 3; Total Protein: $F(9,29) = 50.130$; $p < 0.001$; n = 3). Moreover, it is noteworthy to indicate values from all biochemical parameters increased as the concentration of each drug or the mixture also increased. Thus, the liver function of fish was adversely affected by CPX alone and with PCM in a concentration-dependent manner.

Table 4
Liver function test.

		ALT	ALP	Total Bilirubin	Total Protein
	Reference value	343–410 U/L	0–10 U/L	0.2–0.6 mg/dL	4.4–5.8 g/dL
Control	-	340 ± 9	4 ± 1	0.4 ± 0.1*	4.7 ± 0.3*
PCM	0.125 μg/L	495 ± 15*	13 ± 1*	1.1 ± 0.2*	6.5 ± 0.3*
	0.250 μg/L	547 ± 12*	17 ± 2*	1.5 ± 0.3*	7.1 ± 0.3*
	0.500 μg/L	603 ± 14*	19 ± 1*	1.9 ± 0.3*	7.9 ± 0.2*
CPX	0.250 μg/L	432 ± 11*	11 ± 2*	0.9 ± 0.3*	6.1 ± 0.2*
	0.500 μg/L	504 ± 15*	15 ± 2*	1.3 ± 0.2*	6.8 ± 0.1*
	1 μg/L	538 ± 18*	16 ± 1*	1.4 ± 0.2*	7.0 ± 0.1*
MIXTURES	0.125 + 0.250 μg/L	563 ± 15*	18 ± 1*	1.7 ± 0.3*	6.8 ± 0.2*
	0.250 + 0.500 μg/L	632 ± 17*	21 ± 2*	2.1 ± 0.2*	7.4 ± 0.3*
	0.500 + 1 μg/L	711 ± 16*	26 ± 2*	2.5 ± 0.3*	8.0 ± 0.2*

Data were represented as mean ± SD, n = 3. *: significant difference compared to the control group.

3.2. Oxidative stress

For each treatment group, the antioxidant enzymatic activity of SOD, CAT, and GPx increased in a concentration-dependent manner (Fig. 1). Accordingly, we observed significant differences between all treatment groups except for the lowest concentration of PCM and the middle and highest concentration of CPX in SOD and CAT.

In addition to the increased activity of antioxidant enzymes, all antioxidant biomarkers showed a significant difference compared to the control group (SOD: F (9,29) = 1322.803; $p < 0.001$; n = 3; CAT: F (9,29) = 722.929; $p < 0.001$; n = 3; GPx: F (9,29) = 705.013; $p < 0.001$; n = 3). As in antioxidant enzymes, oxidative damage biomarkers levels increased as the concentration of each of the treatments increased (Fig. 2). In this case, all treatment groups showed significant differences between these and against the control group in all oxidative damage biomarkers (LPx: F (9,29) = 1627.466; $p < 0.001$; n = 3; HPx: F (9,29) = 598 967; $p < 0.001$; n = 3; GPx: F (9,29) = 424.361; $p < 0.001$; n = 3).

3.3. Histopathological damage

Though the prevalence of the tissue alterations increased in a concentration dependent-manner in all treatment groups, these showed significant differences depending on the treatment of fish (Table 5). For example, in fish treated with CPX, we did not find the presence of congestion, hyperemia, and nuclear alterations in the liver of fish. Moreover, at the lowest concentration of this drug, the liver of fish did not show the presence of macrovascular fatty degeneration as well. Accordingly, the livers of fish exposed to CPX got the lowest prevalence percentage compared with PCM and the mixtures. Unlike fish exposed to CPX, we observed the occurrence of nuclear alterations at all concentrations tested, as well as the presence of congestion and hyperemia at the highest concentrations. Concerning mixtures, only fish exposed to the lowest concentrations of these drugs did not show the presence of congestion in the liver, and in consequence, these got the highest prevalence of tissue alterations. In all cases, no matter the prevalence, tissue alterations observed in this study were of the reversible type. However, we believe irreversible tissue alterations are likely to occur if the treatment continues or the concentrations of drugs increase.

Once we got the prevalence of tissue alterations, we then calculated the histopathological index of each concentration from all treatment groups (Table 6). As we aforementioned, to calculate HI, we assigned each alteration with a pathological importance factor (w) and a score value (a). The score value (a) represents the occurrence of the alterations and ranges from 0 to 6, while the pathological importance factor (w) indicates the level of damage in an organ and ranges from 0 to 3. As can be seen in our results, all tissue alterations found in the liver of fish exposed to CPX alone and with PCM got a maximum value of w of 2. Nonetheless, the score value (a) of these alterations was different depending on the concentration and the treatment used in the fish. Thus, the drug with the lowest HI was CPX, followed by PCM and then the mixtures.

Photomicrographs from each treatment group and concentration are shown in Fig. 3. From this figure, we can better appreciate that as the concentration in each of the treatments increases, the tissue alterations in the liver increase as well. Moreover, we can see as well that among the treatments, the mixture is the one that induces the most liver damage compared with the others. For example, from the lowest concentration of the mixtures, we can already see the presence of hyperemia, a tissue alteration that rarely occurred in the other treatments. In addition, in the mixtures, we can appreciate as well that congestion, macrovascular fatty degeneration, and infiltration were more severe than in the other treatments. Thus, mixtures induced more severe damage in the liver of fish than drugs alone.

3.4. Gene expression

Nrf1, Nrf2, BAX, CASP3, CASP6, CASP8, and CAS9 gene expression in the liver of *D. rerio* were affected after the treatment CPX alone and with PCM. For example, after 96 h of exposure, zebrafish adults showed a significant increase in the gene expression of Nrf1 and Nrf2 in all treatment groups compared to the control group (Fig. 4) (Nrf1: F (9,29) = 425.710; $p < 0.001$; n = 3; Nrf2: F (9,29) = 199.367; $p < 0.001$; n = 3). Additionally, similar to Nrf1 and Nrf2, genes involved in the apoptosis process were significantly upregulated in all treatment groups compared to the control group (Fig. 4) (BAX: F (9,29) = 275.611; $p < 0.001$; n = 3; CASP3: F (9,29) = 392.194; $p < 0.001$; n = 3; CASP6: F (9,29) = 440.657; $p < 0.001$; n = 3; CASP8: F (9,29) = 509.160; $p < 0.001$; n = 3; CASP9: F (9,29) = 399.490; $p < 0.001$; n = 3). For all genes, the expression increased in a concentration-dependent manner. Thus, we saw significant differences between concentrations of each treatment but not between all treatment groups. For example, no significant differences were found between concentration 2 of PCM and concentration 3 of CPX in all genes tested.

3.5. Comet assay

As the concentration of CPX alone and with PCM increased, the DNA damage also increased (Fig. 5). Thus, we observed significant differences between the treatment groups. Moreover, we also observed that DNA damage in all treatment groups was significantly different to the control group (F (9,29) = 294.042; p < 0.001; n = 3). Between treatments, the mixtures induced the most severe DNA damage in fish, followed by PCM and CPX, respectively. In Fig. 5, we can also see a representative image from the highest concentration of each treatment (see Fig. 6).

3.6. Micronuclei test

Similar to the comet assay, the proportion of micronuclei in the blood of fish exposed to CPX alone and with PCM increased compared to the control group (F (9,29) = 16.968; $p < 0.001$; n = 3). The number of micronuclei in the blood of fish increased in a concentration-dependent manner. So, we could see significant there were significant differences between the concentrations and between the treatment groups. Once again, CPX showed the lowest damage compared to the other

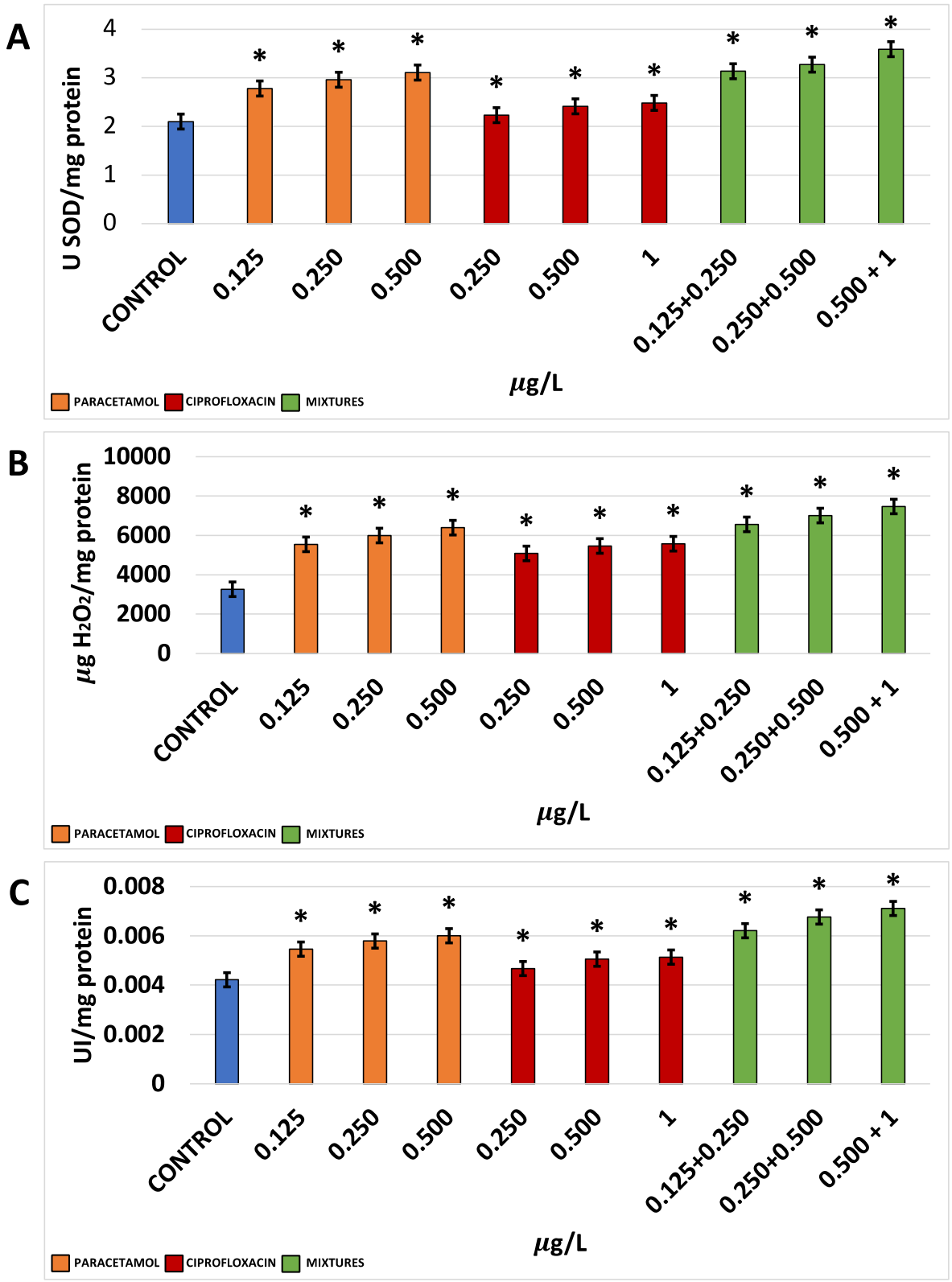

Fig. 1. Enzymatic activity of **A**: SOD, **B**: CAT, and **C**: GPx in the liver of *D. rerio* after 96 h of exposure to CPX alone and with PCM. Data were represented as mean ± SD, n = 3.*: significant difference compared to the control group.

treatments, with a low number of micronuclei observed in the blood of fish exposed to this drug. Moreover, mixtures showed more genotoxic damage in fish than PCM.

3.7. Quantification of PCM, CPX and their mixture

As can be seen in Table 7, after 96 h of exposure, concentrations of both drugs alone and in combination significantly decreased compared to the nominal concentration. Nonetheless, none of the concentrations

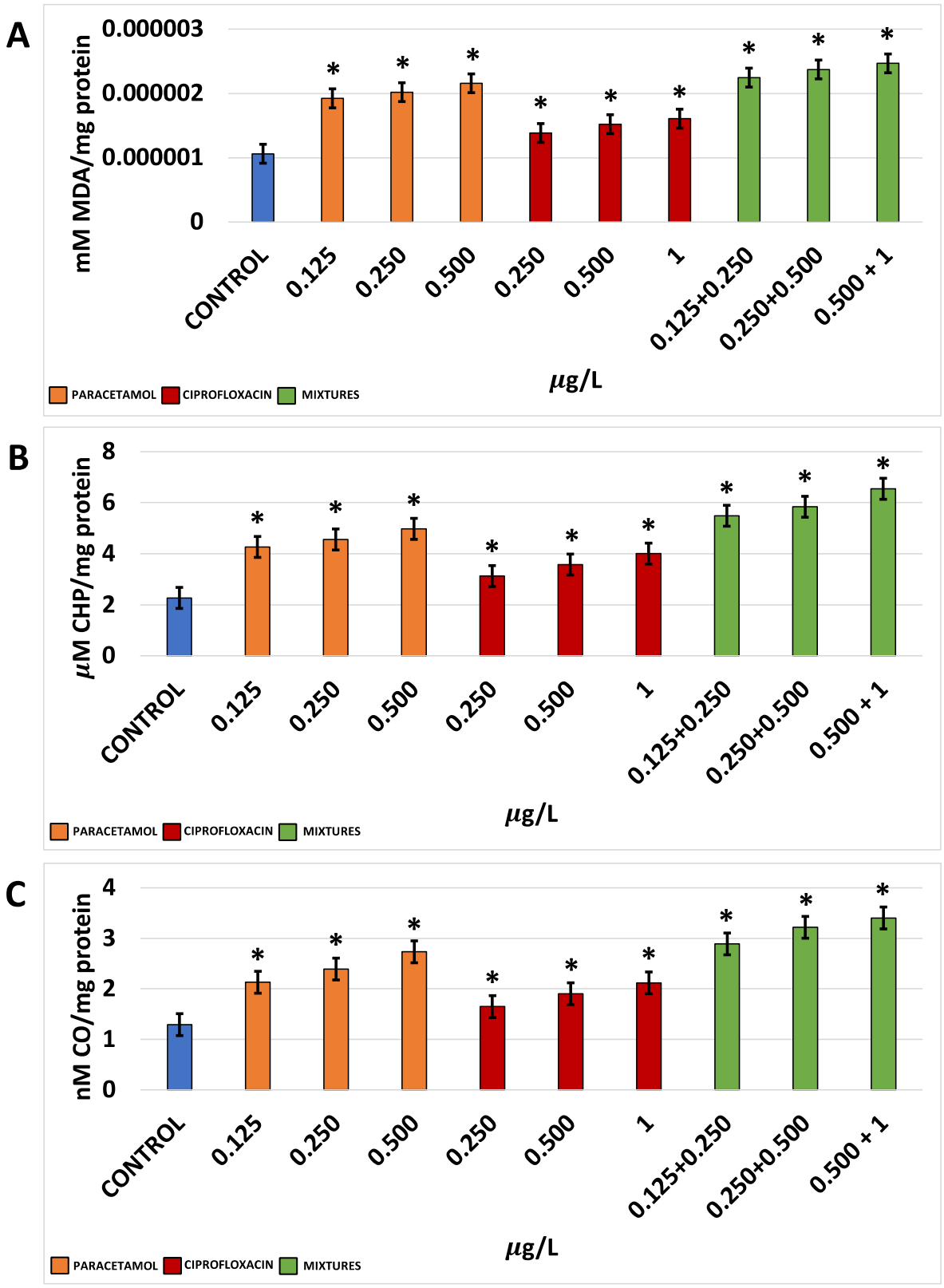

Fig. 2. Levels of **A:** LPx, **B:** HPx, **C:** POx in the liver of *D. rerio* after 96 h of exposure to CPX alone and with PCM. Data were represented as mean ± SD, n = 3. *: significant difference compared to the control group.

decreased more than 20%. Thus, we carried out our analyses based on the nominal concentrations. Concerning drugs quantification in the liver, CPX, and PCM concentrations, individually and in the mixture, that were all above the limit of quantification. Moreover, concentrations of CPX and PCM increased in a concentration-dependent manner in all treatment groups. For the control group, concentrations of both drugs were below the LOQ and did not show any significant differences between the three replicates. Taking into account the nominal

Table 5
Tissue alterations prevalence.

Treatment	Concentration	Tissue alterations							Prevalence
		Congestion	Hyperemia	Macrovascular fatty degeneration	Sinusoidal dilatation	Infiltration	Nuclear alterations	Pyknotic nuclei	
Control	–	0	0	0	0	0	0	1	14.28%
PCM	0.125 μg/L	0	0	1	1	1	1	1	57.14%
	0.250 μg/L	1	0	1	0	1	1	1	71.42%
	0.500 μg/L	1	1	1	0	1	1	1	85.71%
CPX	0.250 μg/L	0	0	0	1	1	0	1	42.85%
	0.500 μg/L	0	0	1	1	1	0	1	57.14%
	1 μg/L	0	0	1	1	1	0	1	57.14%
MIXTURES	0.125 + 0.250 μg/L	0	1	1	1	1	1	1	85.71%
	0.250 + 0.500 μg/L	1	1	1	1	1	1	1	100%
	0.500 + 1 μg/L	1	1	1	1	1	1	1	100%

0: absence of the alteration; 1: presence of the alteration.

concentrations of drugs and the measured concentrations of these in the liver, we estimated the bioconcentration factor of drugs.

Taking into account the measured concentration of the drugs in the water and the liver of fish, we estimated the uptake factor for both compounds. In the case of PCM, the uptake factor ranged from 0.033 to 0.47 g/L, while for CPX, this factor ranged from 0.025 to 0.032 g/L.

3.8. Principal Component Analysis

According to our Principal Component Analysis, there is a strong correlation between all variables tested. For example, in Fig. 7, we can appreciate that the correlation between the prevalence of tissue alterations and oxidative stress biomarkers is robust. Likewise, in the case of gene expression, we can see that these variables showed a close correlation with serum levels of ALT, ALP, TB, and TP.

Moreover, from this figure, we also can see that the mixtures produced more damage in all biomarkers, especially in those related to the prevalence of tissue alterations and the expression of apoptotic genes, compared with the rest of the groups. Concerning PCM, the main biomarkers affected by this drug were those related to biochemical serum levels and the expression of apoptotic genes. Meanwhile, for CPX, it is observed that fish exposed to this drug suffered the mildest damage in all biomarkers compared to PCM and the mixtures.

4. Discussion

Up to date, there is a lot of articles about the toxic effects that PCM may induce in several aquatic species; however, the information concerning the harmful effects that CPX alone and with PCM can produce in water organisms is still limited. Thus, in this study, we aimed to evaluate the hepatotoxic and genotoxic effects that CPX and the mixture of these drugs may induce in *D. rerio* adults.

4.1. Biochemical parameters

According to our results, the liver function of fish was negatively affected by CPX alone and with PCM in a concentration-dependent manner. In agreement with our results, Guo et al., 2015 demonstrated that PCM at concentrations of 2–4 mmol/L increased serum alanine aminotransferase (ALT) levels of zebrafish after 24 and 48 h of exposure. Likewise, Folarin et al., 2018 showed that 128.3 and 12.83 mg/L of PCM boosted the levels of ALT and alkaline phosphatase (ALP) in the liver of catfish (*Clarias gariepinus*) after 28 days of exposure. Concerning CPX, no study we are aware of has studied the toxic effects that this drug may induce on the liver function of fish, but there is a lot of information about this harmful process in rats. Edem et al. (2016), for instance, indicated that ALT and ALP levels in rats exposed to 0.9, 2, and 5 mg/kg/day of CPX for ten days were significantly increased compared to the control group. Moreover, Hemieda et al., 2019 exhibited that serum levels of ALT, AST, ALP, and total bilirubin (TB) in rats treated with 20 mg/kg/day of CPX for ten days significantly augmented. Liver enzymes are responsible for the metabolism and detoxification of all macromolecules in organisms. (Mohafrash and Mohafrash, 2015). The increase in these liver enzymes may be due to liver damage induced by oxidative stress (Hemieda et al., 2019; Chen et al., 2020). However, in the case of CPX, serum levels of enzymes can also be produced by cholestatic pattern, which is basically biliary obstruction (Ahmad et al., 2021).

4.1.1. Oxidative stress

Oxidative stress occurs when reactive oxygen species (ROS) production exceeds the detoxification capacity of these potentially harmful oxidants. In our study, all treatment groups induced an oxidative stress response in liver fish after 96 h of exposure. These results are in agreement with previous findings. For example, Guiloski et al. (2017) showed that PCM at concentrations of 0.25 and 2.5 μg/L caused protein carbonyls and increased SOD activity in *Rhamdia quelen* after 21 days of exposure. Moreover, Nogueira et al., 2019 demonstrated both drugs PCM (0.005–3.125 mg/L) and CPX (0.005–0.488 μg/L) caused oxidative stress in zebrafish embryos after 96 h of exposure. Similarly, Gomes et al., 2017 exhibited that normal electron flow in the respiratory electron transport chain (ETC) and the ability of duckweed (*Lemna minor* L) to cope with hydrogen peroxide were impaired by CPX. About the mixture CPX-PCM, only one previous study demonstrated this mixture could induce oxidative damage in zebrafish embryos after 72 and 96 h of exposure (Rosas-Ramírez et al., 2021). Even though ROS can act as primary or secondary messengers to induce cell death or growth, these may also produce oxidative damage to proteins, cell membranes, and DNA of mitochondria, affecting the mitochondrial capacity to synthesize ATP and to carry out their metabolic functions. Mitochondrial DNA (mDNA) alterations may disturb respiratory chain elements or ribosomal and transfer RNAs, causing several diseases (Tuppen et al., 2010). Moreover, the accumulation of dysfunctional mitochondria may increase cell death rates producing tissue damages (Espada, et al., 2020).

4.2. Gene expression

For PCM particular case, it is known that once ROS are generated, either from mitochondria dysfunction or other sources, these may cause the phosphorylation and activation of many kinases, such as c-jun-*N*-terminal kinase (JNK) (Wu et al., 2017). JNK activated can also phosphorylate several transcription factors, such as p53, NF-kB, and c-Jun, and members of the caspase and Bcl2 families, to accelerate hepatocyte apoptosis (Saberi et al., 2014; Ding et al., 2016). Nonetheless, concerning the apoptosis molecular mechanism of CPX, data still be inconclusive and point out this drug can promote cell death through two different pathways. The first of these pathways implies CPX activates

Table 6
Histopathological index of zebrafish liver.

Treatment	Concentration	Reaction Patterns	Alterations	w	a	HI
Control	–	Circulatory disturbances	Congestion	1	0	0.06
			Hyperemia	1	0	
		Inflammation	Infiltration	2	0	
		Progressive changes	Macrovascular fatty degeneration	1	0	
			Sinusoidal dilatation	1	0	
			Nuclear alterations	2	0	
			Pyknotic nuclei	2	2	
PCM	0.125 μg/L	Circulatory disturbances	Congestion	1	0	0.36
			Hyperemia	1	0	
		Inflammation	Infiltration	2	2	
		Progressive changes	Macrovascular fatty degeneration	1	2	
			Sinusoidal dilatation	1	0	
			Nuclear alterations	2	4	
			Pyknotic nuclei	2	4	
	0.250 μg/L	Circulatory disturbances	Congestion	1	2	0.56
			Hyperemia	1	0	
		Inflammation	Infiltration	2	4	
		Progressive changes	Macrovascular fatty degeneration	1	4	
			Sinusoidal dilatation	1	0	
			Nuclear alterations	2	4	
			Pyknotic nuclei	2	6	
	0.500 μg/L	Circulatory disturbances	Congestion	1	2	0.7
			Hyperemia	1	2	
		Inflammation	Infiltration	2	4	
		Progressive changes	Macrovascular fatty degeneration	1	6	
			Sinusoidal dilatation	1	0	
			Nuclear alterations	2	6	
			Pyknotic nuclei	2	6	
CPX	0.250 μg/L	Circulatory disturbances	Congestion	1	0	0.23
			Hyperemia	1	0	
		Inflammation	Infiltration	2	2	
		Progressive changes	Macrovascular fatty degeneration	1	0	
			Sinusoidal dilatation	1	2	
			Nuclear alterations	2	0	
			Pyknotic nuclei	2	4	
	0.500 μg/L	Circulatory disturbances	Congestion	1	0	0.33
			Hyperemia	1	0	
		Inflammation	Infiltration	2	4	
		Progressive changes	Macrovascular fatty degeneration	1	2	
			Sinusoidal dilatation	1	2	
			Nuclear alterations	2	0	
			Pyknotic nuclei	2	4	
	1 μg/L	Circulatory disturbances	Congestion	1	0	0.36
			Hyperemia	1	0	
		Inflammation	Infiltration	2	4	
		Progressive changes	Macrovascular fatty degeneration	1	4	
			Sinusoidal dilatation	1	2	
			Nuclear alterations	2	0	
			Pyknotic nuclei	2	4	
MIXTURES	0.125 + 0.250 μg/L	Circulatory disturbances	Congestion	1	0	0.6
			Hyperemia	1	4	
		Inflammation	Infiltration	2	4	
		Progressive changes	Macrovascular fatty degeneration	1	4	
			Sinusoidal dilatation	1	4	
			Nuclear alterations	2	2	
			Pyknotic nuclei	2	6	
	0.250 + 0.500 μg/L	Circulatory disturbances	Congestion	1	2	0.76
			Hyperemia	1	4	
		Inflammation	Infiltration	2	4	
		Progressive changes	Macrovascular fatty degeneration	1	4	
			Sinusoidal dilatation	1	4	
			Nuclear alterations	2	6	
			Pyknotic nuclei	2	6	
	0.500 + 1 μg/L	Circulatory disturbances	Congestion	1	4	0.83
			Hyperemia	1	4	
		Inflammation	Infiltration	2	4	
		Progressive changes	Macrovascular fatty degeneration	1	4	
			Sinusoidal dilatation	1	6	
			Nuclear alterations	2	6	
			Pyknotic nuclei	2	6	

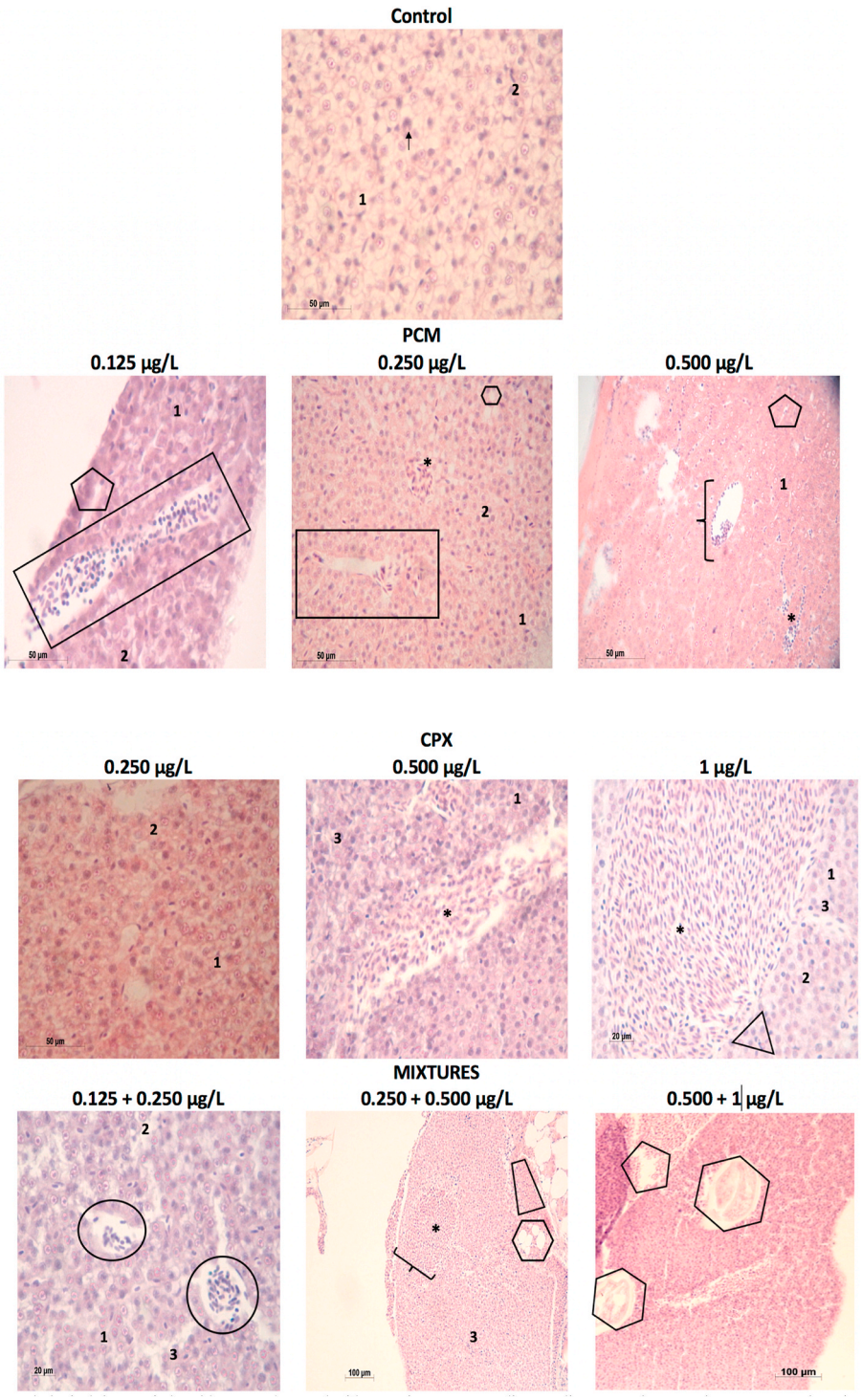

Fig. 3. Histopathological damage induced by CPX alone and with PCM in *Danio rerio* liver. **1:** liver parenchyma; **2:** hepatocytes; **3:** hepatic sinusoids; {: central vein; ↑: Pyknotic nuclei; *: infiltration;: portal vein portal vein;: macrovascular fatty degeneration;: sinusoidal dilatation;: nuclear alterations;: hyperemia;: congestion.

topo-II mediated DNA, which in turn activates ATM/p53 pathway leading to apoptosis (Yadav and Talwar, 2019), while the other via involve mitochondrial depolarization by CPX, with subsequent alteration in Bax: Bcl2 ratio, and finally PARP cleavage (Herold et al., 2002; Yadav and Talwar, 2019). In this study, we demonstrated CPX alone and with PCM induced a significant increase in the gene expression of Nrf1, Nrf2, BAX, CASP-3, CASP-6, CASP-8, and CASP-9. Analogously, Sijia et al.2019 showed PCM (5–5000 µg/L) significantly induced the gene expression of Nrf1 and CAT in Daphnia manga at 96 h of exposure. Moreover, Koagow et al., 2020 demonstrated PCM at a range of concentrations 40 ng/L to 100 µg/L altered the gene expression of HSP70, CASP8, BCL2, and FAS in the gonads of blue mussels (*Mytilus edulis*). No previous studies had demonstrated CPX altered the gene expression of apoptosis- and oxidative stress-related genes in fish until ours. Nonetheless, several previous studies have shown this drug may upregulate the expression of BAX, BCL2, CASP7, CAS9, SOD2, GPX3, p53, HSP70, and TPT1 in different cell cultures and earthworms (Beberok et al., 2018; Salimiaghdam et al., 2020; Yang et al., 2020; Alaaeldin et al.,

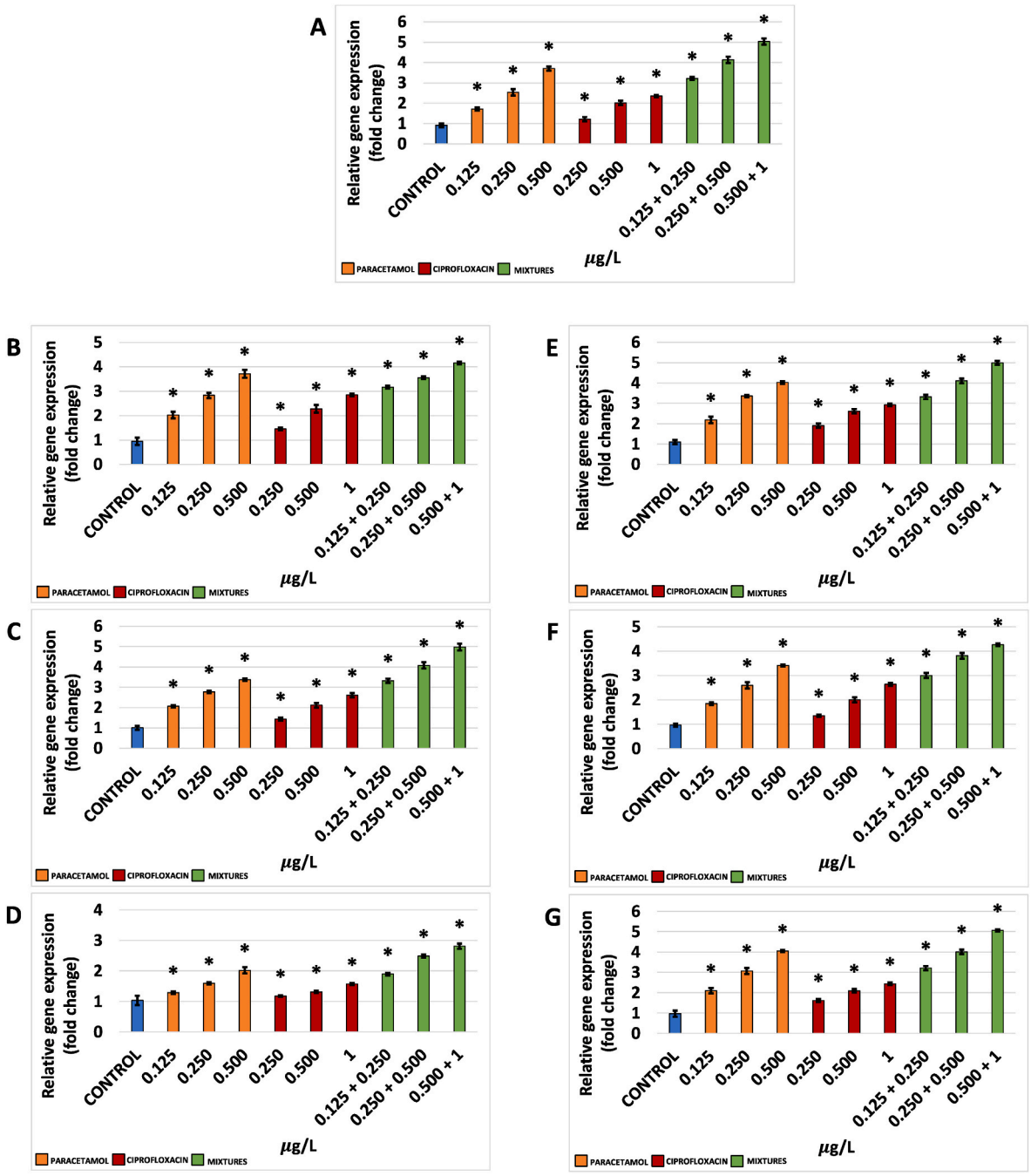

Fig. 4. Effects of different concentrations of CPX alone and with PCM on **A**: Nrf1, **B**: Nrf2, **C**: BAX; **D:** CASP3, **E:** CAPS6, **F:** CASP8, and **G:** CASP 9 genes in *D. rerio* at 96 h. Data were represented as mean ± SD, n = 3. *: significant difference compared to the control group.

2020).

4.3. Histopathological damage

Apoptosis is responsible for the physiological removal of undesirable cells, as well as tissue remodeling during development. However, due to the low rates of cell turnover in the liver and the quick and continuous elimination of cells, apoptosis may also be responsible for liver tissue damage (Guicciardi et al., 2013). Moreover, since accumulating evidence has demonstrated that hepatic apoptosis and oxidative stress play a central role in CPX-induced hepatotoxicity (Adikwu and Brambaifa, 2012; Adikwu and Deo, 2012; Wu et al., 2017; Guiloski et al., 2017; Hemieda et al., 2019), these two pathways may be responsible for the tissue alterations observed in our study. According to our results, CPX alone and with PCM induced congestion, hyperemia, macrovascular fatty degeneration, sinusoidal dilatation, infiltration, nuclear alterations, and pyknotic nuclei formation in the liver fish. In agreement with these results, Gamal et al., 2017 demonstrated that PCM, even at low doses, disrupts the integrity of cell-cell tight junction adhesions, which led to liver injury in the human hepatic HepaRG cell line. In addition, Guiloski et al. (2017) pointed out *Rhamdia quelen* fish exposed to 0.25 and 2.5 µg/L of PCM for 21 days showed mild blood congestion and leucocytes infiltration. Finally, concerning CPX, Ramesh et al., 2021 found several tissue alterations, such as lamellar fusion, necrosis, epithelial lifting, and cytoplasmic vacuolation in the gill, liver, and kidney tissues of *Cirrhinus mrigala* treated with 1 and 1.5 µg/L of this drug for 15 days.

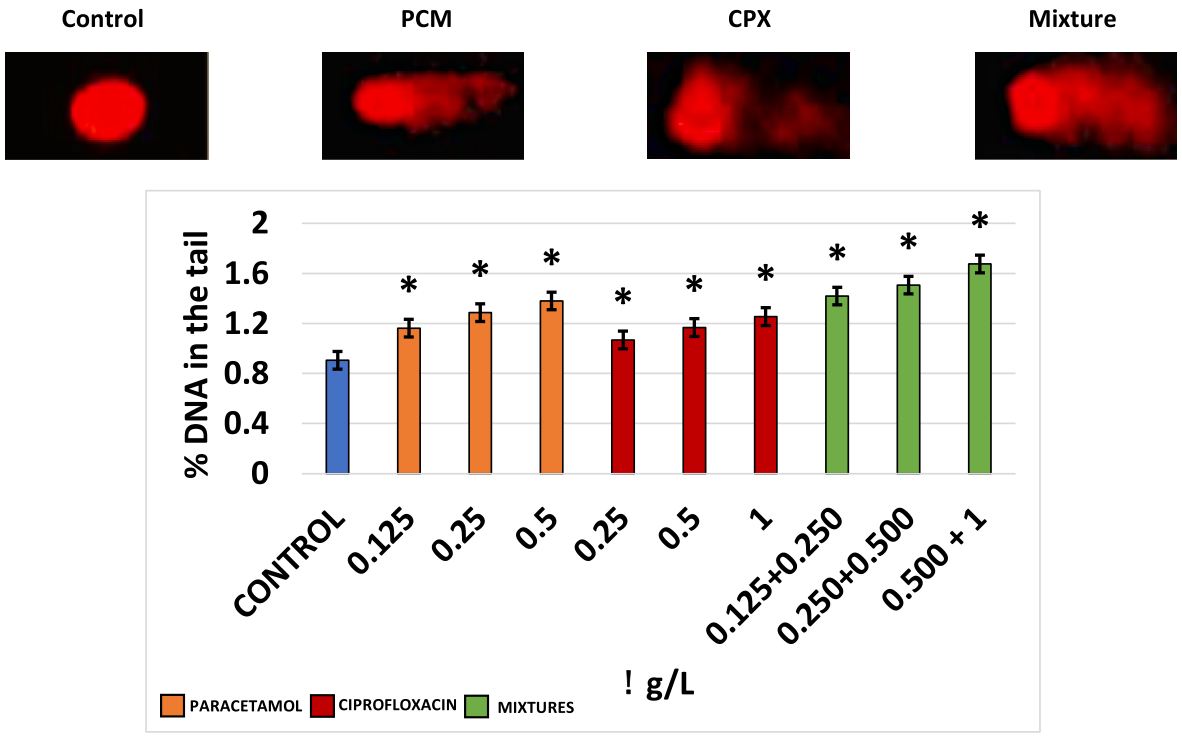

Fig 5. DNA damage in blood cells of *D. rerio* exposed to CPX alone and with PCM. Data were represented as mean ± S/D, n = 3. *: significant difference compared to the control group.

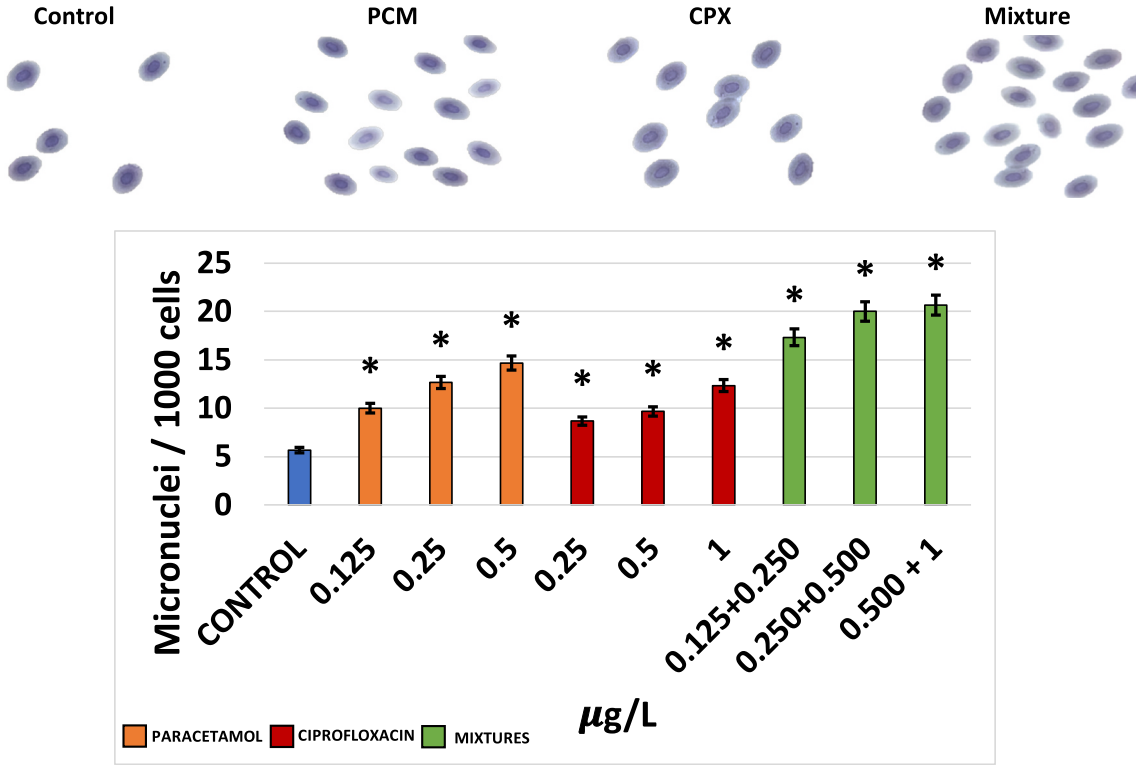

Fig. 6. Frequency of micronuclei in blood cells of *D. rerio* exposed to CPX alone and with PCM. Data were represented as mean ± SD, n = 3. *: significant difference compared to the control group.

4.4. Genotoxicity

ROS do not only are related to apoptosis and tissue alterations but also DNA damage. Moreover, CPX-induced genotoxicity could also be related to its capacity to inhibit topoisomerase I and IV (Rosas-Ramírez et al., 2021). To our knowledge, our results are the first evidence of CPX-induced genotoxicity in fish. In agreement with our results, previous studies in HepG2 cells and two different plants (Vicia faba and

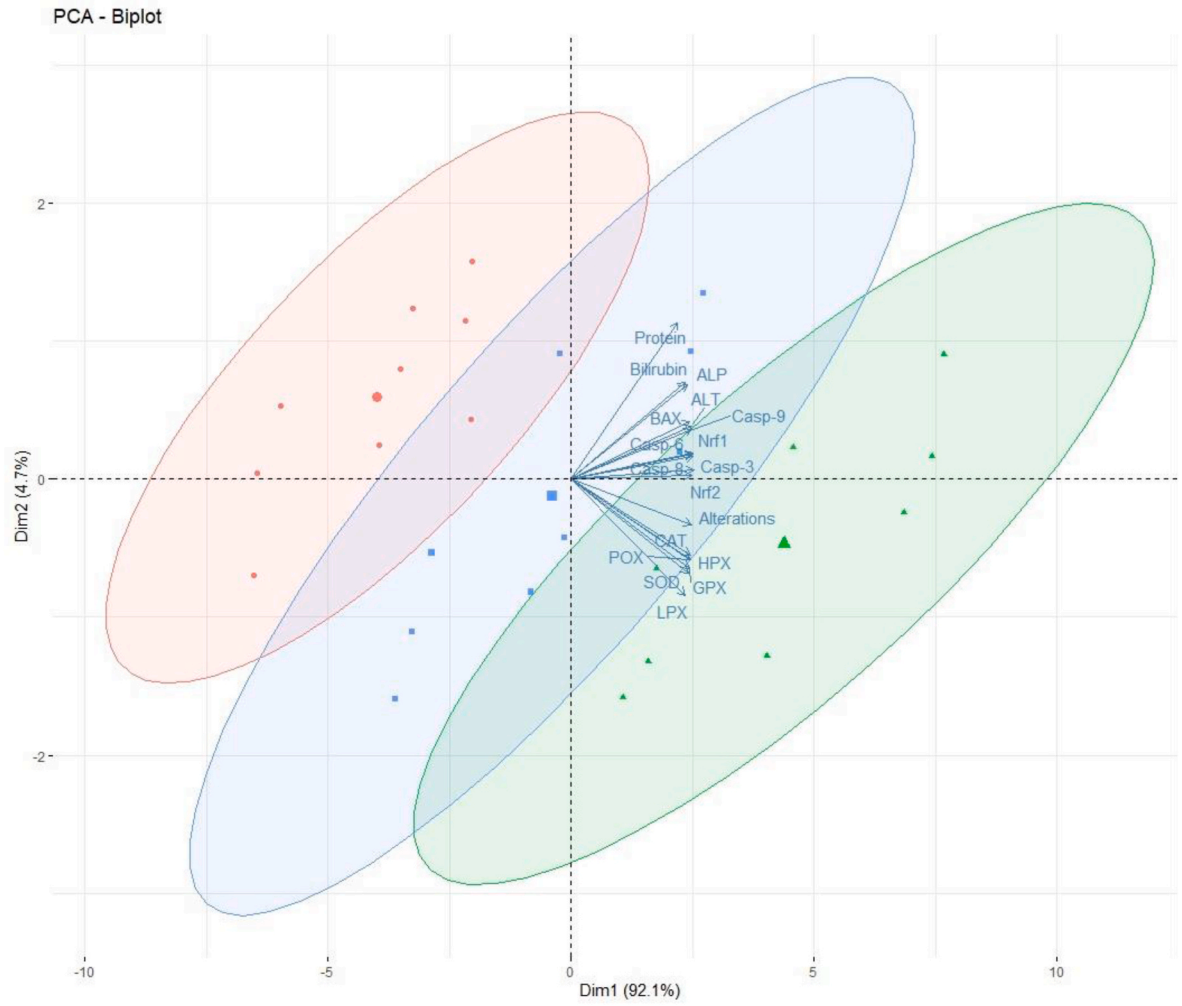

Fig. 7. Principal Component Analysis of D. rerio biomarkers evaluated after exposure to PCM, CPX, and their mixture.

Table 7
Concentrations of PCM, CPX, and their mixture in the liver of zebrafish.

	Nominal concentration	Measured concentrations
Quantification in water		
Control	ND	<LOQ
PCM	0.125 μg/L	106 ng/L
	0.250 μg/L	202 ng/L
	0.500 μg/L	421 ng/L
CPX	0.250 μg/L	217 ng/L
	0.500 μg/L	434 ng/L
	1 μg/L	912 ng/L
MIXTURES	0.125 + 0.250 μg/L	110 + 218 ng/L
	0.250 + 0.500 μg/L	207 + 439 ng/L
	0.500 + 1 μg/L	428 ng/L + 921 ng/L
Quantification in liver		
Control	ND	<LOQ
PCM	0.125 μg/L	5 ng/g
	0.250 μg/L	9 ng/g
	0.500 μg/L	14 ng/g
CPX	0.250 μg/L	7 ng/g
	0.500 μg/L	12 ng/g
	1 μg/L	23 ng/g
MIXTURES	0.125 + 0.250 μg/L	4 + 7 ng/g
	0.250 + 0.500 μg/L	6 + 11 ng/g
	0.500 + 1 μg/L	10 + 21 ng/g

ND: not detected; PCM: LOQ: limit of quantification (10 ng/L and 2 ng/g) and LOD: limit of detection (5 ng/L and 1 ng/g); CPX: LOQ: limit of quantification (8 ng/L and 5 ng/g) and LOD: limit of detection (5 ng/L and 3 ng/g).

Allium cepa) have demonstrated CPX induced genotoxicity (Garcia-Käufer et al., Khadra et al., 2012; Borba et al., 2018). Moreover, several other studies have reported PCM-induced genotoxicity in cultured cells, fish, and rats (Salah et al., 2012; Wasborg, 2016; Perussolo et al., 2019).

4.5. *Mixtures and mechanism of toxicity*

As can be seen in our results, mixtures were more harmful to the fish liver than drugs alone, which may be due to the joint metabolism of drugs. PCM molecule is not in itself associated with toxicity. Instead, *N*-acetyl-*p*-benzoquinoneimine (NAPQI), a highly reactive intermediate metabolite, is responsible for PCM-related toxicity (Mian et al., 2020). NAPQI is formed after the oxidative metabolism of PCM, and the CYP1A2, CYP2E1, and CYP3A4 isoenzymes are the main ones responsible for its formation (Kalsi et al., 2011). Rosas-Ramírez et al., 2021 pointed out that CYP1A2-inhibition by CPX could be related to an increase in the serum concentration of non-metabolized PCM and NAPQI. The above, because cumulative data suggest CYP1A2 does not play a significant role in NAPQI generation as CYP2E1 does (Tonge et al., 1998; Hazai et al., 2002; Snawder et al., 1994). Nonetheless, another author showed that concomitant administration of PCM increased the extent absorption of CPX, and at the same time, decreased its rate of absorption (Issa et al., 2007). Thus, more studies are needed to understand the drug-drug interaction between PCM and CPX.

Another explanation for higher damage induced by the PCM-CPX

mixtures in liver fish may be related to their toxicity mechanism. PCM, for instance, is known to induce its toxic effects by its primary metabolite, NAPQI. NAPQI in organisms causes glutathione depletion by activation of glutathione-S-transferase (Yoon et al., 2016; Tittarelli et al., 2017). Moreover, due to its ability to bind thiol groups, this metabolite produces the formation of protein adducts with cysteine and lysine residues of hepatocytes' mitochondrial proteins (Jaeschke et al., 2012a,b; Tittarelli et al., 2017). Thus, the excessive presence of NAPQI in organisms results in oxidative stress, ATP stores depletion, and mitochondrial dysfunction. Mitochondrial dysfunction then may lead to homeostasis alteration, an increase in cell membrane permeability, vacuolization, and the loss of cellular elements (Jaeschke et al., 2012). On the other hand, the CPX mechanism of toxicity is not well understood. Some researchers have stated that the formation of ROS by CPX in the microsomal system might provide an explanation for the hepatotoxicity observed after exposure to this drug (Hincal and Taskin, 1995; Gürbay et al., 2001; Weyers et al., 2002). Thus, ROS generated by CPX may lead to depletion of protein content, which subsequently may result in a significant decrease in the number and degeneration of mitochondria (Gu and Manautou, 2012). Alterations of proteins and lipids have also been seen in cells suffering apoptosis in reaction to non-oxidative stimuli, such as in the case of topoisomerase II inhibitors (Slater et al., 1995). Fluoroquinolones have been described to DNA binding resulting in a marked inhibition of DNA topoisomerase (Adikwu and Brambaifa, 2012). This mechanism could buttress as an additional mechanism used by CPX to suppress DNA and protein synthesis, alter intracellular cAMP and calcium levels, and induce mitochondrial damage in cells of the liver (Adikwu and Deo, 2012). Thus, we believe that if the latter described mechanism of CPX combines with the PCM mechanism, the hepatotoxic effect of both compounds could be exacerbated. Nonetheless, more studies are needed to understand the toxic mechanism of the CPX-PCM mixture.

Drugs may exert their toxic effects in sensitive species, either under favorable conditions of uptake or long durations of exposure (Nunes et al., 2015; Nunes, 2020). In our study, we demonstrated fish were able to uptake CPX and PCM from the medium, and though the uptake of these drugs by the fish was low, fish showed significant alterations in their liver function. Similar to our results, Burket et al. (2020) demonstrated fish (Campostoma anomalum) and mussels (Corbicula fluminea) were able to uptake PCM from the water of a municipal effluent. Moreover, Zhao et al. (2015) showed CPX bioaccumulated in bile, plasma, liver, and muscle tissues of wild fish from the water of highly urbanized region of China. Thus, CPX is not only absorbed by fish but reaches different organs and damage these.

5. Conclusion

To the best of our knowledge, this is the first study that demonstrates the hepatotoxic and genotoxic effects of the CPX alone and with PCM in non-target organisms. According to our results, CPX alone and with PCM altered the serum levels of ALT, ALP, TP, and TB of fish after 96 h of exposure. Moreover, different tissue alterations were observed in the liver of fish exposed to all treatments. Concerning genotoxicity, we demonstrated CPX alone and with PCM induced DNA damage and increased the frequency of micronuclei in fish. Hepatotoxic and genotoxic effects in fish may be related to the oxidative stress response. The above because our results showed that the activity of SOD, CAT, and GPx, as well as levels of LPx, HPx, and POx in liver fish, were increased after treatment with CPX alone and with PCM. Also, oxidative stress induced by CPX alone and with PCM induced upregulation of genes related to apoptosis (BAX, CASP3, CASP6, CASP8, and CASP9) and oxidative stress (Nrf1 and Nrf2), which may further buttress the hepatotoxic and genotoxic response of CPX.

Gustavo Axel Elizalde-Velázquez and Jonathan Ricardo Rosas-Ramírez, José Manuel Orozco-Hernández and Karina Rosales-Pérez performed all the exposure experiments. Leobardo Manuel Gómez-Oliván and Gustavo Axel Elizalde-Velázquez were involved in the conception, Leobardo Manuel Gómez-Oliván and Gustavo Axel Elizalde-Velázquez, Sandra García-Medina and Hariz Islas Flores were involved in the design and interpretation of the data and the writing of the manuscript with input from Demetrio Raldua, Xochitl Guzmán-García and Marcela Galar-Martínez.

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.

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