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Survival and malformations rates, oxidative status in early life stages of *Cyprinus carpio* due to exposure to environmentally realistic concentrations of paracetamol

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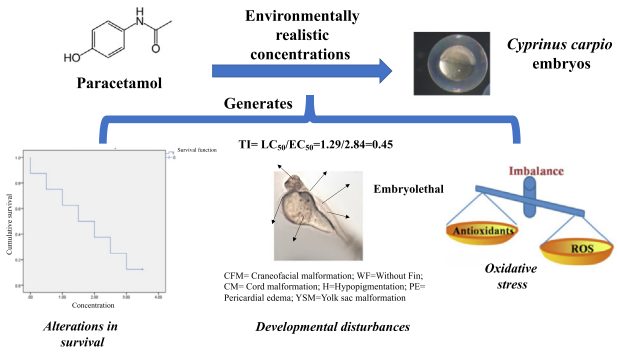
HIGHLIGHTS

- Survival and malformation rates induced by paracetamol was evaluated in *Cyprinus carpio*.
- Oxidative stress status was evaluated at 72 and 96 hpf in embryos of *Cyprinus carpio*.
- Paracetamol reduced the survival rate of the embryos of up to 90%.
- At concentrations of 2.0–3.5 μg/L of paracetamol the most severe malformations occurred.
- Paracetamol alters embryonic development and oxidative status in *Cyprinus carpio*.

GRAPHICAL ABSTRACT



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ABSTRACT

Paracetamol (PCM) is among the most consumed analgesic and antipyretic drugs worldwide. Due to its high consumption, this drug has been reported ubiquitously on different water bodies, posing a real threat to aquatic organisms. Until now, several studies have pointed out that PCM may induce oxidative stress, histological damage and developmental disorders on different aquatic species. Nonetheless, there is still a huge knowledge gap about the toxic effects that PCM may induce in species of commercial interest such as the common carp *Cyprinus carpio*. The aim of this study was to evaluate survival and malformation rates induced by PCM (0.5 μg/L – 3.5 μg/L) in early life stages of common carp. Furthermore, oxidative stress biomarkers were evaluated at 72 and 96 h post fecundation. PCM reduced the survival rate of the embryos of up to 90%, as concentration increased. LC_{50} and EC_{50m} were 1.29 μg/L and 2.84 μg/L, respectively. Biomarkers of cellular oxidation and antioxidant enzymes were modified in a concentration-dependent way with respect to the control group ($p < 0.05$). The main developmental alterations observed were lordosis, scoliosis, craniofacial malformations, hypopigmentation, growth retardation, pericardial edema and rachyschisis. These data indicate that environmentally realistic concentrations of PCM could be hazardous and affects the development in early stages of *C. carpio*. Moreover, our findings also indicate that *C. carpio* embryos may be a useful *in vivo* model to evaluate embryonic and teratogenic effects of drugs such as PCM.

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1. Introduction

Paracetamol (PCM) also known as acetaminophen (*N*-acetyl-p-aminophenol) is a drug that possesses analgesic and antipyretic properties. This drug is mainly used in the treatment of osteoarthritis, especially hand, hips, shoulders, and knees (Anderson, 2008). However, it is also prescribed for gastric and duodenal ulcers, gastritis and hiatal hernia, as an alternative treatment in case of salicylates allergies and in patients with hemophilia or receiving anticoagulants (Acevedo-Barrios et al., 2017). Furthermore, because PCM does not require a prescription for its consumption, it is usually utilized to improve cold and flu symptoms (Li et al., 2015).

The pharmacological activity of PCM is associated with the inhibition of the active site of the isoforms 1 and 2 of cyclooxygenase (COX) enzymes (Smith, 2009). In addition, PCM has a variant of COX-1 which is prostaglandin H2 synthetase called COX-3 (Hersh et al., 2005). The new splice variant of COX-1 called COX-3, recently discovered in the brain of dogs (Chandrasekharan et al., 2002), is sensitive to paracetamol and it is believed that plays an important role in the biosynthesis of prostanoids known as important mediators of pain and fever. Drugs that preferentially block COX-1 also appear to act on COX-3. However, at the human nucleotide sequence level, the existence of COX-3 is questionable (Hazarika and Selvam, 2019).

Thanks to its efficacy and relative high safety, PCM is one of the best-selling pain relievers worldwide. In 2015, 85.6% of the yearly worldwide production was concentrated in Asia, specifically in China and India with a 64.4 and 21.2% respectively. Furthermore, some data indicates that in 2019 worldwide PCM sales summed a total of 740 million USD and it is forecasted that by 2023 its sales will rise to 780 million USD (Acetaminophen Global Market Report, 2018).

As a consequence of its intense usage and partial degradation (14–75%), large amounts of PCM and its toxic transformation by-products are incorporated into different environmental matrices such as soil, rivers, drains, leachates, effluents from wastewater treatment plants, water tables, drinking water from which their levels ranged the ng/L to μg/L (Joss et al., 2006; Murray et al., 2010; De Luna et al., 2012; Kawabata et al., 2012; Kunkel and Radke, 2012; Nunes et al., 2014; Li et al., 2015; Petrie et al., 2015; Rivera-Jaimes et al., 2018). Table 1 summarizes some PCM occurrence data in different countries around the world.

As can be seen in Table 1, the worldwide occurrence of PCM on different water matrices vary from one country to other. This may be explained due to the metabolism of PCM, the design used in the wastewater treatment plants and the proportion of treated waters incorporated into surface waters (Kunkel and Radke, 2012).

Although, PCM is not a highly persistent compound in the environment, its continuous aggregation into the medium exceeds its transformation rate, producing adverse effects on aquatic organisms (Ratola et al., 2012). For instance, Choi et al., 2018 demonstrated that concentrations of 10 and 30 μg/L of this drug may induce histological damage to the gills, structural damage to the kidney and decrease the glycogen levels in the liver of *Oncorhynchus mykiss*, after 4 weeks of exposure. Additionally, Liu et al. (2019) showed that PCM may alter the expression of Nrf1, gene related to the anti-oxidant system and redox homeostasis disruption, in *Daphnia magna*. These results are in agreement with our previous findings, where PCM induced oxidative stress and genotoxicity in *Hyalella azteca*, *Daphnia magna* and *C. carpio* at concentrations of 770 μg/L, 100 μg/L and 246 μg/L, at 72, 48 and 96 h, respectively (Gómez-Oliván et al., 2012, 2014a, 2014b; Nava-Álvarez et al., 2014). Therefore, the adverse effects induced by PCM in aqutic organisms are related to the increased production of reactive oxygen species (ROS) during its biotransformation process.

Since ROS are capable of producing oxidative stress and this phenomenon has been associated with embryotoxic and teratogenic effects in non-target organisms (Gutiérrez-Noya et al., 2020; El Hajam et al., 2020; Luja-Mondragón et al., 2019), PCM may induce embryotoxicity in aquatic organisms. Although, previous studies have reported that PCM induced embryotoxic and teratogenic effects on *Danio rerio* and *Xenopus laevis* (Cedron et al., 2020; Fort et al., 1992; Saide et al., 2019). These findings were identified at other concentrations of PCM different from those used in this work. Furthermore, in these works the relationship between these alterations and oxidative stress was not demonstrated. The novelty of this work is that embryotoxic and teratogenic effects induced by PCM were identified for the first time at environmentally relevant concentrations, identified in several water bodies of the world. Besides, the effects were demonstrated in a species of commercial interest in many countries of Latin America and Asia as the common carp *Cyprinus carpio*. This work together with the above mentioned, complement the information of the ecotoxicological profile of PCM, a drug that is considered relatively safe. Therefore, it was hypothesized that the PCM at environmentally realistic concentrations would exhibit alterations in survival, malformation rates and oxidative stress in early stages of common carp *C. carpio.*

2. Material and methods

2.1. Chemicals

Paracetamol (*N*-acetyl-4-aminophenol, CAS number: 103–90-2) with linear formula $CH_3CONHC_6H_4OH$ was purchased from Sigma-Aldrich (Mexico), with an analytical purity of ≥99%. All other compounds used were purchased from Sigma-Aldrich (Mexico), unless it is indicated in the undergoing method at any given point of the experiment.

2.2. Test organisms and maintenance

The test organisms used in this study were provided by the Tiacaque Carpicola Center located in the municipality of Jocotitlán, State of Mexico. This center is the main producer of common and herbivorous carp in the country. The carps used for the study were organisms in a reproductive age with in a size of 45 to 60 cm and weighting between 5 and 7.5 Kg.

The test organisms were placed in breeding ponds for 20 days prior to the experiments, separating the males from the females. Water parameters were strictly controlled: temperature was maintained at 23 ± 2 °C, conductivity at 740 ± 100 μS/cm, pH at 7.0 ± 1.0 and dissolved oxygen ≥95% saturation. The carps were kept in a photoperiod (12:12 h) and fed daily with protein supplemented food (35%), enriched with carbohydrates and other nutrients to ensure that the carps improve the desired husbandry index. A day before the experiment, the male carps were joined with the female in the spawning ponds in order to obtain eggs.

2.3. Egg production

C. carpio eggs were obtained through an *in situ* fertilization process using the natural method. *C. carpio* embryos in the blastula stage (2 h post-fertilization) were collected immediately after natural mating, rinsed in water, and checked under a stereomicroscope (Zeiss Stemi 305). The unfertilized eggs and those showing cleavage irregularities or injuries were discarded.

The fertilization process was carried out in spawning ponds of 25–30 m^2 in which ten males and five females were placed. To favor the fertilization process, the females were previously injected with human chorionic gonadotropin (hCG-500 IU/Kg) in the caudal vein. At the bottom of the spawning ponds, nets and casuarina branches were placed in order to retain the oocytes. The embryos in blastula period were exposed to environmentally realistic concentrations of PCM. The selection of realistic concentrations of PCM used in this study was made considering studies of occurrence in freshwater bodies around the world, which is where common carp, *C. carpio* can survive (Archer

Table 1
Occurrence of PCM in different types of effluents and substrates in water bodies.

Country	Types of effluents and substrates in water bodies PCM Concentrations (μg/L)						References
	WWTP Influent (min. – max.)	WWTP Effluent (min. – max.)	Surface Water (min. – max.)	Drinking Water (min. – max.)	Sludge (min. – max.)	Hospital (min. – max.)	
Canada	36–500	0.16–62	NA	NA	0.014–0.015	NA	(Guerra et al., 2014)
China (Shanghai)	NA	NA	0.075	NA	NA	NA	(Yao et al., 2018)
China (Xiamen city)	7.9×10^6	4.37×10^6	NA	NA	NA	NA	(Wang et al., 2018)
Czech Republic	0.35–180	<0.01–13	NA	NA	NA	NA	(Vymazal et al., 2017)
India	Min 21.126 ± 1.671 Max 134.322 ± 32.419	Min ND Max 0.058 ± 0.012	NA	NA	NA	NA	(Mohapatra et al., 2016)
Mexico (Cuernavaca)	2.3–14.9	NA	NA	NA	NA	NA	(Rivera-Jaimes et al., 2018)
Portugal (Coimbra)	0.081–9.286	0.083–0.106	NA	NA	NA	University: 13.03–58.86 General: 12.56–47.14 Pedriatric: 2.27–57.143 Maternity: 0.211–13.986	Santos et al., 2013
Portugal (Rivers)	NA	1.63–3.57	Paracetamol - Glucuronide up to 462 p - aminophenol below 1.63	NA	NA	NA	
Republic of Korea	3.540–10.234	0–0.027	NA	NA	NA	NA	(Behera et al., 2011)
Saudi Arabia	99.6	90.5	NA	NA	NA	NA	(Shraim et al., 2017)
South Africa (North West province)	21.27–119.50	ND - 11.39	NA	NA	NA	NA	(Kanama et al., 2018)
South Africa (Rivers)	NA	NA	Downstream 0.064 Upstream 0.021	NA	NA	NA	(Archer et al., 2017)
South Korea	NA	NA	NA	NA	90.3 ng/g industrial 56.6 ng/g domestic 68.5 ng/g mixed	NA	(Subedi et al., 2014)
Sweden (Stockholm)	NA	NA	0.159	NA	NA	NA	(Lindim et al., 2019)
United Kingdom	NA	NA	55	NA	NA	NA	(Bound and Voulvoulis, 2006)
United States of America (Georgia)	NA	NA	37–130	NA	NA	NA	(Yang et al., 2011)
United States of America (Long Island)	NA	N	Execution rocks: 0.029 Light house: 0.07 Railway bridge: 0.06 Daymark: 0.09	NA	NA	NA	(Cantwell et al., 2019)
United States of America (Southeastern)	NA	NA	Max 0.192	NA	NA	NA	(Padhye et al., 2014)

NA = not available; ND = not detected; WWTP = wastewater treatment plant.

et al., 2017; Bound and Voulvoulis, 2006; Yang et al., 2011; Cantwell et al., 2019). As well as other studies carried out by our group (Madín reservoir), that the PCM concentration varies in the places where the common carp usually lives (Pérez-Coyotl et al., 2019).

2.4. Embryo-survival test

C. carpio embryos were exposed to seven graded concentrations of the PCM at levels of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 μg/L, prepared by successive dilutions of stock solution. These test systems were processed together with a PCM-free control system. The test systems consisted of 24-well microplates, to which a randomly selected fertilized oocyte was placed, to form batches of 20 oocytes for each PCM concentration tested. The tests were performed in triplicate. The microplates were maintained for 96 h at a temperature of 24 ± 1 °C and during natural light-dark photoperiods in the laboratory. 60 embryos were observed for each PCM concentration tested using a Zeiss Stemi 305 stereoscopic microscope. Observations were made at 96 hpf, and lethality was considered when coagulated oocytes occurred or when no heartbeat was detected. At the end of the test, the number of dead embryos, survivors and those with malformations were recorded for each concentration of PCM. LC_{50} and EC_{50m} values were determined by means of a maximum likelihood linear regression in addition to their respective 95% confidence intervals ($p < 0.05$). This analysis was performed using the US-EPA software ver 1.5, according to the Trimmed Spearman-Karber method (Hamilton et al., 1977). The data obtained was used to calculate the teratogenic index (TI), which is the relationship between LC_{50}/EC_{50m}. The interpretation of the TI was taken into account using as a reference the criteria established by Weigt et al. (2011), which considers that if the TI is greater than 1, the PCM would be identified as a teratogenic compound; but if it is less than 1 then this compound would be embryolethal.

2.5. Fish embryo-toxicity test (FET)

Fish embryo toxicity test was based on the OECD guideline Protocol 236 "Fish Embryo Toxicity" (FET) test (Test No. 236: Fish Embryo Acute Toxicity (FET) Test, 2013). Some adaptations to the test were made according to Luja-Mondragón et al. (2019). *C. carpio* embryos were exposed to seven graded concentrations of the PCM at levels of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 μg/L, prepared by successive dilutions of stock solution. These test systems were processed together with a PCM-free control system. The test was performed using 60 eggs per treatment, divided in 3 replicates, selected and distributed in 24-well microplates in bioclimate chamber, 20 wells were filled with 2 mL of the test solution and four wells with water (internal plate control, as required in the OECD guideline). The test was started immediately after fertilization and up to 96 h. The embryos were observed at 12, 24, 48, 72 and 96 h post-fertilization (hpf) using the Zeiss Stemi 305 stereomicroscope. The development parameters of the embryos were evaluated throughout the test period using × 70 magnification for the eggs and × 40 for the hatched embryos. Before hatching, the following parameters were evaluated: egg coagulation, otolith formation, general delay in development, eye and body pigmentation, somites formation, heartbeat, edemas, detachment of the tail-bud from the yolk sac, yolk sac absorption and hatching. The alterations were established considering the criteria established by Hermsen et al. (2011) and Kimmel et al. (1995) and with the consequent modifications for the common carp referred by Luja-Mondragón et al. (2019).

2.6. Morphology scoring system and assessment of teratogenicity

The systems cited in Section 2.5 were used for this purpose. The evaluation of *C. carpio* eggs and larvae exposed to environmentally relevant concentrations was performed at 12, 24, 48, 72 and 96 hpf. For each alteration to embryonic development, a score was assigned considering the criteria established by Hermsen et al. (2011) and Kimmel et al. (1995) and with the consequent modifications for the common carp referred by Luja-Mondragón et al. (2019). Acknowledging the alterations to embryonic development and the teratogenic disorders generated by the presence of PCM, a frequency histogram was charted out in which the main drug induced malformations triggered on the *C. carpio* eggs and embryos were quantified after the exposure to this drug.

Alterations such as detachment of tail, somite formation, eye development, movement, heartbeat, blood circulation, head-body pigmentation, tail pigmentation, pectoral fin, protruding mouth and hatching were registered. These alterations were recorded at the demarcated check points (12, 24, 48, 72 and 96 hpf) and a score was assigned at each given time according to Luja-Mondragón et al., 2019.

2.7. Oxidative stress biomarkers evaluation

One gram of *C. carpio* eggs (average 1150 ± 250 embryos) were exposed to PCM concentrations of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 μg/L in six-liter systems. After 72 and 96 h, the embryos were homogenized with phosphate buffer at pH 7.4 and the cell oxidation biomarkers were determined: lipoperoxidation level (Buege and Aust, 1978), hydroperoxide content (Jiang et al., 1992) and antioxidant enzymes superoxide dismutase and catalase by Misra and Fridovich (1972) and Radi et al. (1991) methods, respectively. These methods are performed routinely in our facilities and can be reviewed in detail at Morachis-Valdez et al., 2015; Saucedo-Vence et al., 2015. The experiments were replicated fivefold. The exposure times selected to determine the biomarkers of oxidative stress were 72 and 96 hpf, these were chosen because the zebrafish larvae had already hatched and their enzymatic system was already working properly.

2.8. Ethical approval

This protocol was reviewed and approved by the Ethics Committee of the Universidad Autónoma del Estado de México (UAEM) to ensure that it was carried out in accordance with institutional standards for the care of animal subjects (Approval ID: ME.AUSMCF.REC.123.2019). Provisions in the official Mexican norm on breeding, care and use of laboratory animals (NOM-062-ZOO- 1999) were also taken into account.

2.9. Statistical analysis and test reliability criteria

For the calculation of the EC_{50} malformations and LC_{50} values, US-EPA software ver 1.5 method was used, as dictated by the Trimmed Spearman-Karber, through probit analysis with maximum likelihood linear regression. To characterize the teratogenic potential of a test substance, the teratogenicity index (TI) was calculated using the ratio of LC_{50} and EC_{50}. Furthermore, the survival data was analyzed by constructing a survival graph using a Kaplan-Meier analysis employing the IBM SPSS Statistics 22 software.

A Student's *t*-test (one-tailed) was performed to identify statistically significant differences between treatment and controls groups ($p < 0.05$, IBM SPSS Statistics 22 software). Statistics were done on the basis of affected embryos (embryos with lethal or teratogenic effects); at first, the scores for each concentration of PCM had to be registered, then for the control were gathered and finally a comparison was completed using the t-test.

The results of the oxidative stress biomarkers were examined using a two-way analysis of variance (ANOVA) considering concentration and exposure time followed by a Tukey-Kramer multiple comparisons test with a 95% confidence limit, in order to determine the differences between the means. For reaching such results we resource to the IBM SPSS Statistics 22 software.

To guarantee the traceability of the results, two main validating criteria for the test were established; the first one was to ensure a fertilization percentage ≥ 90% and the second one, for the test to be

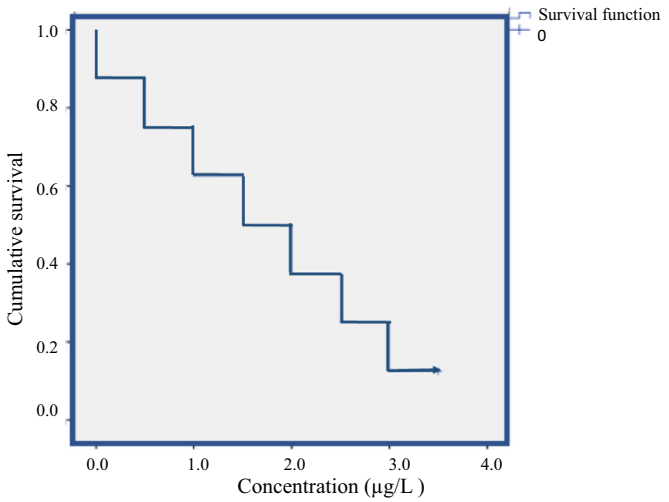

Fig. 1. Survival graph by Kaplan-Meier analysis in *C. carpio* by PCM exposure.

considered valid, an analysis of the control group at the 96 hpf mark was conducted in order to review that it did not present a lethal teratogenic effect index greater than 10%.

3. Results

3.1. Embryo survival test

For all the concentrations of PCM, mortality and malformations data was concentration- dependent ($p < 0.05$). The LC_{50} (for embryotoxic effects/lethality) and EC_{50} (for particular teratogenic effects) data were obtained at 96 hpf for the concentration-response curve on a minimum of 3 separate experiments. The LC_{50} value was 1.29 µg/L with 95% confidence intervals between 1.13 and 1.44 µg/L. EC_{50m} value was 2.84 µg/L with intervals between 2.38 and 3.65 µg/L and the value TI was 0.45. According to the criteria of Weigt et al. (2011), PCM was embryolethal.

Fig. 1 shows a Kaplan-Meier graph, in which the median OS can be observed, which was 1.7 µg/L, the lower limit 0.95 and the upper 2.54 µg/L. Survival diminishes reaching its highest expression at the concentration of 2.5 µg/L. At 3 and 3.5 µg/L, a slight increase in survival is observed respect to 2.5 µg/L ($p < 0.05$). The malformations augmented depending on the concentration up to 2 µg/L. Mortality growth dose-dependently, reaching its maximum at the concentration of 3.5 µg/L.

3.2. Teratogenic alterations

Several abnormalities such as growth retardation, pericardical edema, skeletal alterations were observed in *C. carpio* embryos exposed to all the concentrations of PCM ($p < 0.05$).Pericardial edema, craniofacial malformations, skeletal alterations (scoliosis, lordosis, kyphosis and rachyschisis) and hatching retardation were elicited in the common carp embryos exposed to PCM from 2.5 and up to 3.5 µg/L respect to control group ($p < 0.05$) [Fig. 2]. In many of the embryos exposed to PCM at concentrations above 2.5 µg/L, death was observed after 96 h of exposure, possibly due to severe malformations presented at these concentrations.

3.3. Morphology scoring evaluation and assessment of teratogenicity

Fig. 3. shows the concentration–response curves for morphological score performed from all PCM concentrations. For PCM, there was a clear shift between concentration-response curves, suggesting a progressive effect on the development of alterations in all concentrations tested. Among all morphological features, skeletal alterations and growth retardation were the most affected. Concentrations from 2.5 µg/L showed significant differences in each PCM concentration and exposure time in comparison to the control ($p < 0.05$). Concentrations between 1.0 and 2.0 µg/L, exhibited very similar scores in the embryonic development of *C. carpio*; however, these concentrations were significantly different in contrast to the control group ($p < 0.05$). When comparing the scores obtained at 0.5 and 1.0 µg/L concentrations, a statistically significant reduction was observed among 42% and 50% in parallel to the concentrations of 3 and 3.5 µg/L ($p < 0.05$). The greatest alterations in the development of embryos exposed to PCM were observed at 3.5 µg/L.

Despite the different concentrations at which PCM exerted their effects, the patterns of teratogenic effects appeared very similar (Fig. 4), mostly comprising head and heart malformations, scoliosis, yolk deformation and edema in exposed embryos. The control group did not present any teratogenic alterations in neither exposure time. The

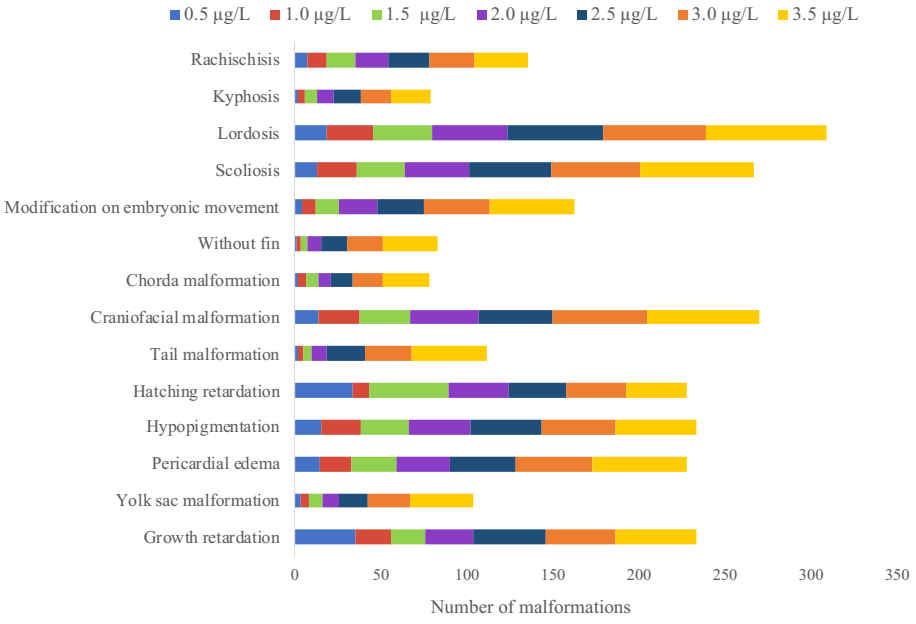

Fig. 2. Main malformations induced by exposure to PCM concentrations in *C. carpio* embryos.

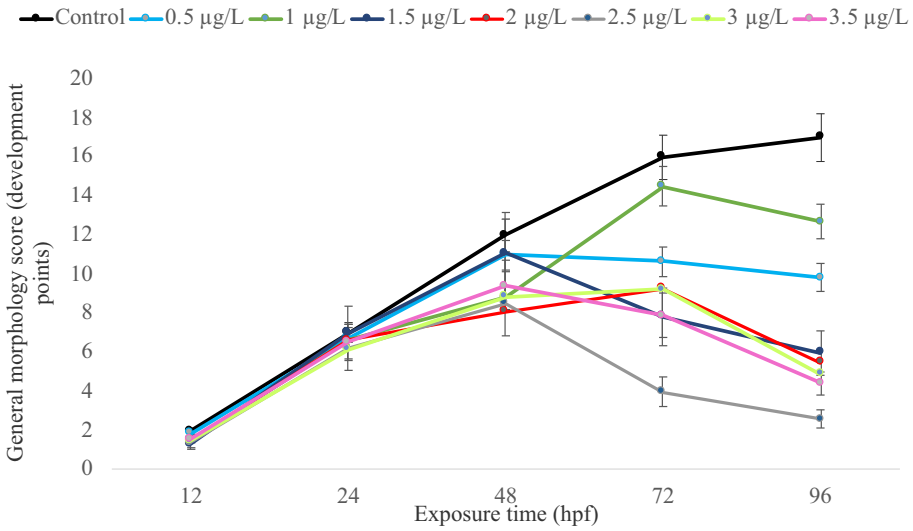

Fig. 3. Concentration-response curves of PCM in *C. carpio* embryos. After 48 h, all PCM concentrations were significantly different from the control group ($p < 0.05$). The differences are not indicated so that the figure can be easily appreciated (Student's *t*-test).

concentrations of 2.5, 3.0 and 3.5 µg/L generated the most serious malformations mainly at 72 and 96 hpf. These malformations were lordosis, scoliosis, craniofacial malformations, hypopigmentation, growth retardation, pericardial edema and rachyschisis.

3.4. Oxidative stress evaluation

All oxidative stress biomarkers showed statistically significant differences compared to the control group ($p < 0.05$) at the two exposure times (Table 2). The maximum alterations observed in the biomarkers of oxidative stress were presented at the concentration of 3.5 µg/L. At 3.5 µg/L, were observed increments with respect to the control group of 136% and 80.9% in HPC, 97.7% and 98% in LPX, 322% and 341% in SOD activity, and 125% and 114.9 in CAT activity, at 72 and 96 h, respectively ($p < 0.05$). All PCM concentrations produced oxidative damage in embryos exposed at 72 and 96 h except for 1 µg/L for HPC and SOD activity, and for 2 µg/L for LPX and for 3 µg/L for HPC ($p > 0.05$).

4. Discussion

This research reveals that *C. carpio* embryos are a suitable model to predict PCM teratogenicity. Although, there are some other studies that had identified PCM-induced alterations in embryonic development, the used concentrations of this NSAID are higher than those identified in water bodies. In addition, the above mentioned studies have shown teratogenic effects by PCM on organisms such as *Xenopus laevis* and *Danio rerio* and not on a species of commercial interest such as *C. carpio* and environmentally relevant concentrations of PCM. The findings identified in this study made possible to establish the argument that PCM in environmentally relevant concentrations is capable of causing alterations in embryonic development, teratogenic effects and as well as affect the survival of *C. carpio* embryos. Furthermore, these findings are related to alteration in oxidative status in carp embryos.

The toxic effects of PCM have been identified in various aquatic organisms such as *Hyalella azteca, Phorcus lineatus* and *Oncorhynchus mykiss* in different studies (Almeida and Nunes, 2019; Choi et al., 2018). The main identified effects of PCM have been related to its ability for inducing oxidative stress in aquatic organisms (Barbosa et al., 2020; Piedade et al., 2020; Liu et al., 2019; Nunes et al., 2017; Gómez-Oliván et al., 2012). The oxidative stress induction capacity has been associated with the PCM biotransformation process, once it undergoes through this activity it generates ROS that are capable of oxidizing biomolecules like lipids and proteins (Almeida and Nunes, 2019; Gómez-Oliván et al., 2012). Excessive production of ROS can overwhelm the innate antioxidant response, leading to pro-oxidant conditions in the cell and generating lipoperoxidation, DNA damage, disruption of signaling cascades, irregular gene expression or altered protein function and degradation of existing proteins (Gómez-Oliván et al., 2012).

N-acetyl-p-benzoquinoemine (NAPQI), an extremely toxic intermediary compound, is produced during the oxidation process of PCM by cytochrome P450 enzymes (Xu et al., 2008). Once this metabolite is conjugated with glutathione, it is eliminated from the human body (Kavitha et al., 2011), and spillage on to water bodies, where can induce toxic effects on non-target organisms. (Barbosa et al., 2020; Nunes et al., 2017). Other ways, in which PCM can induce oxidative stress is by activating the Apoptosis Inducer Factor (AIF), which works like NADH oxidase and regulates the activity of the mitochondrial I complex (Jaeschke et al., 2018). Furthermore, PCM may induce the production of perooxynitrite (ONOO-) a very potent and highly reactive oxidant. Perooxynitrite is generated in the mitochondria of hepatocytes and sinusoidal endothelial cells and forms nitrotyrosine protein adducts. The perooxynitrite formed in the mitochondria leads to the nitration of proteins present in that organelle such as SOD2 (Jaeschke et al., 2012).

Redox signaling is vital to embryogenesis. For instance, during the developmental period there are changes in redox potential that guide cell fate towards either proliferation in a reduced state or differentiation, apoptosis, or necrosis with an increased oxidative state. Furthermore, it is known that proliferative processes, differentiation and apoptotic activity are regulated somewhere by redox states and that changes in this or its imbalance can alter them. It is considered that the most susceptible periods in embryonic development are those that define the transition from one important event to another, (for example: from proliferation to differentiation) in the development of a certain organ or system. Therefore, a substance that is capable of inducing oxidative stress for long periods, characterized by redox imbalance may alter the normal development (Jensen et al., 2001).

The toxicity induced by PCM in *C. carpio* embryos can also be explained by the drug action mechanism, which blocks COX enzymes, responsible for the degradation of arachidonic acid for the production of prostaglandins (PGs) (Bazan et al., 2012). These cell mediators are involved in a wide variety of physiological functions of various species, including an important role in neuronal transmission and ion transport across cell membranes (Arkhipova et al., 2006). The modification of the ion exchange through the chorion can be reflected in a delay in the growth of *C. carpio* embryo and altering the hatching process.

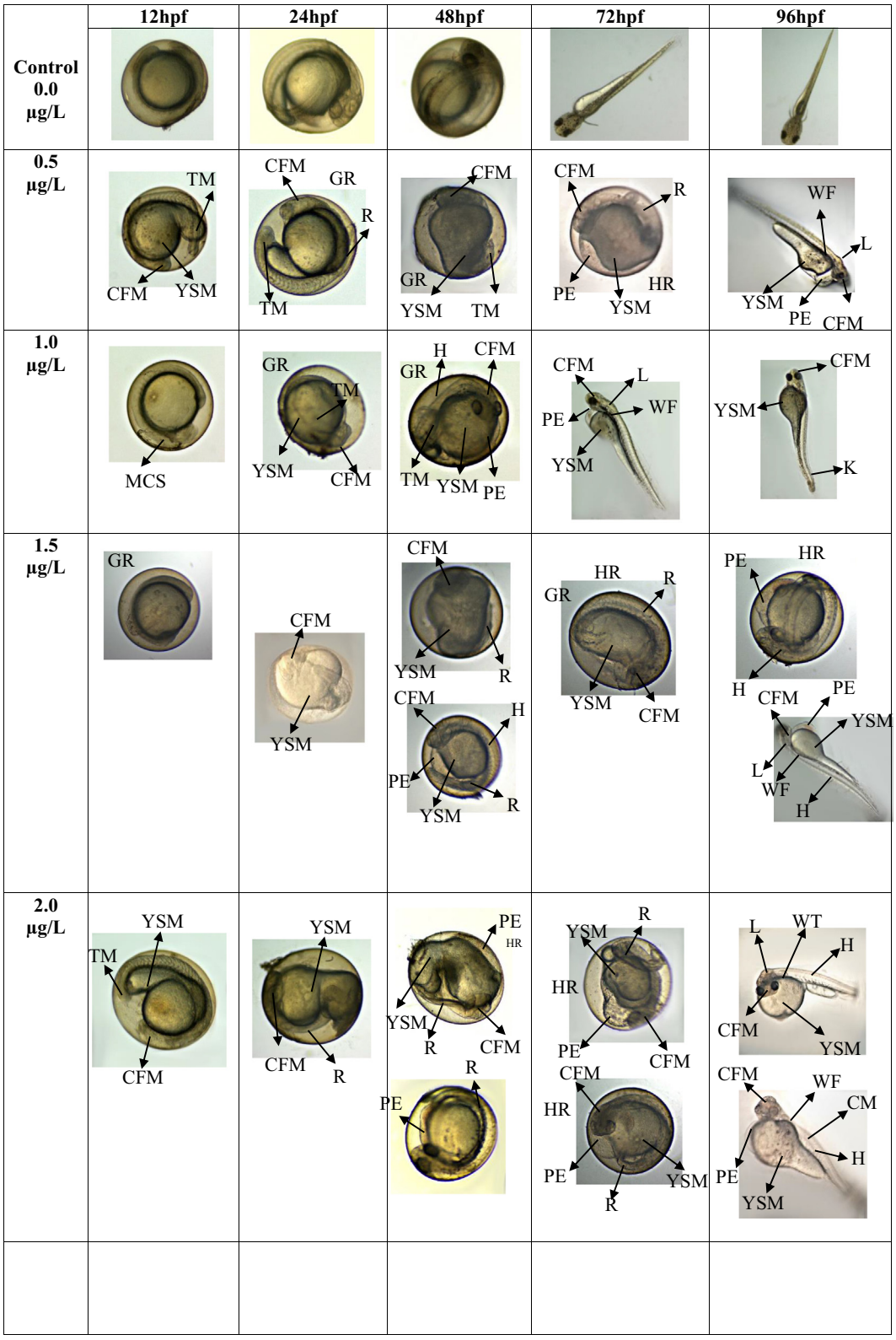

Fig. 4. Teratogenic effects of PCM exposure on morphological features in *C. carpio* embryos. Abbreviations: CFM = Craneofacial malformation, R = Rachischisis, L = Lordosis, HR = Hatching retardation, YSM = Yolk Sac Malformation, WF=Without Fin, S=Scoliosis, H=Hypopigmentation, PE = Pericardial edema, CH = Cord hemorrhage, TM = Tail Malformation, K=Kyphosis.

In this study, we corroborated that PCM is capable of inducing oxidative stress in common carp embryos in a concentration dependent manner. Furthermore, at 72 and 96 h, this drug at all concentrations tested was capable to increase the levels of LPX, HPC, and the enzymes SOD and CAT activity with respect to the control group ($p < 0.05$). In agreement with these results, concentrations of 5 mM of PCM may generate oxidative stress due to a reduction in 47.9% of glutathione (GSH) in embryos of *Xenopus laevis* (Saide et al., 2019).

Table 2
Biomarkers of cellular oxidation and antioxidation in *C. carpio* embryos at 72 and 96 h exposed to PCM.

Concentration µg/L	HPC nM of CHP/mg PT		LPX mM MDA/mg PT		SOD IU SOD/ mg PT		CAT µM H_2O_2/mg PT	
	72 hpf	96 hpf	72 hpf	96 hpf	72 hpf	96 hpf	72 hpf	96 hpf
Control (0)	0.58 ± 0.01	0.63 ± 0.01	58.3 ± 0.6	61.4 ± 0.5	0.27 ± 0.01	0.29 ± 0.3	87 ± 1.1	94 ± 1.3
0.5	0.74 ± 0.02*,a,b	0.76 ± 0.02*,a,b	61.2 ± 0.5*,a,b	65.8 ± 0.7*,a,b	0.35 ± 0.05*,a,b	0.41 ± 0.06*,a,b	103 ± 1.9*,a,b	109 ± 1.4*,a,b
1.0	0.81 ± 0.01*,a,b	0.83 ± 0.03*,a,b	73.6 ± 0.7*,a,b	76.5 ± 0.4*,a,b	0.47 ± 0.06*,a,b	0.49 ± 0.05*,a,b	115 ± 1.4*,a,b	121 ± 1.6*,a,b
1.5	0.94 ± 0.02*,a,b	0.96 ± 0.02*,a,b	82.6 ± 0.4*,a,b	85.6 ± 0.3*,a,b	0.56 ± 0.06*,a,b	0.61 ± 0.04*,a,b	137 ± 0.6*,a,b	141 ± 0.4*,a,b
2.0	1.02 ± 0.04*,a,b	1.07 ± 0.05*,a,b	94.6 ± 0.9*,a,b	96.3 ± 0.7*,a,b	0.65 ± 0.04*,a,b	0.71 ± 0.04*,a,b	141 ± 1.4*,a,b	151 ± 1.5*,a,b
2.5	1.17 ± 0.02*,a,b	1.21 ± 0.03*,a,b	101.8 ± 1.0*,a,b	107.7 ± 1.2*,a,b	0.85 ± 0.04*,a,b	0.92 ± 0.07*,a,b	157 ± 1.9*,a,b	165 ± 2.5*,a,b
3.0	1.29 ± 0.02*,a,b	1.31 ± 0.03*,a,b	108.7 ± 1.1*,a,b	112.5 ± 1.0*,a,b	0.98 ± 0.06*,a,b	1.05 ± 0.05*,a,b	171 ± 1.9*,a,b	182 ± 1.6*,a,b
3.5	1.37 ± 0.02*,a,b	1.41 ± 0.04*,a,b	115.3 ± 0.8 *,a,b	121.6 ± 1.0*,a,b	1.14 ± 0.04*,a,b	1.28 ± 0.06*,a,b	196 ± 1.6*,a,b	202 ± 2.1*,a,b

ANOVA and Tukey-Kramer.
CHP = cumene hydroperoxide; MDA = malondialdehyde.
Values are the mean of five replicates ± SEM.
* Significantly different ($p < 0.05$) from the control group.
[a] Significantly different with respect to time.
[b] Significantly different with respect to concentration.

Nrf1 protein and its isoforms, are indispensable for maintain cellular homoeostasis, normal organ development, and growth during different life stages (Zhang and Xiang, 2016). Furthermore, Nrf1 proteins have the capacity to maintain the antioxidant defences and respond to oxidative stress (Timme-Laragy et al., 2018). Thus, these proteins could work as transcription factors for a considerable number of genes that take part in the glutathione synthesis and Redox signaling (Sant et al., 2017).

At the early life-stages of embryonic development in various aquatic organisms like fish, there is a high susceptibility to oxidative damage by ROS (Parlak, 2018; Wu and Zhou, 2012). The imbalance of oxidative status is caused by an aerobic metabolism overload due to the high energy demand that occurs in the embryo growth process. Other conditions that can also lead to the imbalance of oxidative status are: 1) free radicals elevated production, 2) incremented iron levels not bounded to proteins (NPBI) and 3) antioxidant systems lacking maturity (Mohd Zanuri et al., 2017; Petitjean et al., 2019).

The results of the embryoletality test showed that the malformations in the carp embryos were more severe at higher concentrations of PCM. Furthermore, the highest percentages of embryo death were observed at concentrations of 2–3.5 µg/L (Fig. 1). The observed effects of death can be explained by the NAPQI presence within the embryo. In cyprinids, hepatocytes are known for forming at 32 hpf, but the embryos liver starts to function at 72 h after hatching (Chu and Sadler, 2009; Driessen et al., 2013). Therefore, embryo registered deaths were caused by the NAPQI toxic effect which were related to the concentration exposure. In addition to NAPQI, ROS that form during phase I biotransformation of PCM such as superoxide anion O_2^* and H_2O_2 that are highly oxidizing, can destabilize the proteins and lipids of *C. carpio* larvae affecting their functions and causing embryos death (Parolini et al., 2010; Parvez and Raisuddin, 2005).

Once the PCM passes through the chorion and ROS are formed inside the larvae of *C. carpio*, these oxidize the lipids and bind to the proteins, favoring the generation of embryotoxic effects (Weil et al., 2009).

When evaluating the malformations that were induced by PCM at different environmentally relevant concentrations and different exposure times, we observed that the most common malformations were lordosis, scoliosis, craniofacial malformations, hypopigmentation, growth retardation, pericardial edema and rachyschisis. These malformations could be explained by the presence of ROS and the NAPQI. The increase of ROS within the *C. carpio* embryo can alter intracellular calcium homeostasis, which leads to DNA damage (Kang et al., 2012) and cellular apoptosis that generates genotoxic, cytotoxic, embryotoxic and morphological alterations (Larsson et al., 2016; Shen et al., 2019). In addition, the increase in ROS in oocytes has been associated with reduced fertilization success, diminished embryo quality and increased abnormalities which can be observed in the later life of the offspring (Lord and John Aitken, 2013). It is also known that the oxidation of lipids by the presence of ROS can inhibit the osteoblasts differentiation and promote osteoclasts differentiation through the increasing expression of interleukin-6 (Tseng et al., 2010), these effects contributed to the bone resorption causing skeletal alterations (Boglione et al., 2013) like the ones observed in our study.

Since PCM is a highly soluble drug (0.1 mg/mL) and has a high biotransformation capacity (approximately 90%), this drug can permeate the chorion pores (0.5–0.7 µm) on fertilized eggs while these ones are in the gastrula stage (Rawson et al., 2000). During the embryos development, the chorion permeability changes encouraging a greater penetration as the development time increases. This has been shown in a study done by Kim et al. (2004), who identified that a greater penetration force is necessary to pierce the chorion in blastula and gastrula stages than in the pre-hatching stage embryos. Also, Kais et al. (2013), demonstrated that the permeability of 2,7-dichlorofluorescein was greater with increasing embryos development time, being higher at 48 hpf than at 24 hpf.

In this study, we observed that craniofacial malformations, lordosis, scoliosis and rachyschisis are the most common, occurring malformations at all evaluated exposure times and in all tested concentrations. These results are consistent with the investigation carried out by Cedron et al. (2020), who demonstrated that PCM at concentrations 2.5, 3.5, 4.9, 6.9, 9.6, and 13.4 mM may produce craniofacial abnormalities which are dose-dependent in zebrafish and that this drug also favors the sox9b gene expression reduction. These authors concluded that PCM affects proliferation, composition and joint formation of differentiated cranial neural crest cells.

These craniofacial alterations could also be explained through the effect that the formed NAPQI in PCM metabolization process being capable of binding to the G proteins present in the embryo, those are responsible for regulating the chondrocytes proliferation which are the skeletal system precursors (Cohen et al., 2014).

Another identified alteration that affects the embryonic development identified in our study was hypopigmentation. These results are similar to those found by Cedron et al. (2020), who detected a decrease in pigmentation in zebrafish embryos exposed to 2.5 and 6.9 mM of PCM and melanin synthesis blockage. They also found that zebrafish embryos exposed to 4.9 µM PCM suffered from a decrease in melanin content at 48, 72 and 96 hpf of 83.9, 65.1 and 60.3% respectively. In addition, in another study it was reported that PCM at a concentration of 3.3×10^{-4} mM is capable of reducing the pigmentation of *Danio rerio* embryos (David and Pancharatna, 2009). It is well known that pigmentation in zebrafish embryos begins at 25 hpf in the retinal epithelium and then spreads throughout the skin (Fernandes et al., 2016). In our study, hypopigmentation of *C. carpio* embryos was observed at

concentrations of >1.0 μg/L, after 48 hpf. With these results we could can conclude that as it was shown with zebrafish, PCM blocks melanin synthesis *in C. carpio* embryos. Moreover, as indicated by Cedron et al. (2020), PCM could interfere with the development of black pigment cells; also, PCM might act directly preventing tyrosine biotransformation in order to produce melanin (Vad et al., 2009).

In the present study, pericardial edema was also identified in *C. carpio* at all tested PCM concentrations. This same outcome was found on *Xenopus laevis* frogs when exposed to 100 and 150 mg/L of PCM (Fort et al., 1992). Similarly, in a study conducted by Weigt et al. (2011), it was determined that concentrations of 4 and 6 mM of PCM induced pericardial edema on *Danio rerio* embryos. This malformation could be explained by the increase of apoptotic cells observed by Cedron et al. (2020) in zebrafish embryos exposed to 2.5 and 4.9 mM PCM during 72 and 96 hpf. Some studies have identified that cellular apoptosis is responsible for cellular damage and malformations in the early life stages of aquatic organisms (AnvariFar et al., 2017; Elmore, 2007; Sawant et al., 2014).

The growth retardation found in this study can also be explained by the increase of ROS generated by PCM exposure in the embryos. The excess of ROS can generate the inhibition of choriolysins (proteases) that are necessary to digest the chorion. Likewise, increasing ROS can inhibit hatching enzyme 1 (HE1), which is necessary to break down the chorion. During the 72 hpf, the weakening of the chorion is important, since this favors the first spontaneous movements of the larvae, to leave this membrane and begin the development (Haendel et al., 2004). Inhibition of the previously mentioned enzymes may explain the growth retardation. Similarly, in the embryo growth process, the oxygen requirements are high in the oocytes, so an imbalance in the redox status can generate growth retardation (Pašková et al., 2011).

The growth retardation may also be related to the mechanisms of PCM action, which acts as a blockage for cyclooxygenase. Studies conducted by Cha et al. (2005, 2006); showed that inhibition of cyclooxygenase 1 (COX-1) causes defective formation of intersomitic vascularization and growth arrest. These authors reported that produced prostaglandins by COX-1 activation are necessary in the processes of gastrulation and segmentation.

PCM at concentrations of 1–100 mg/L has been associated with growth retardation manifested along with delayed hatching, reduced body mass and tail malformation on zebrafish embryos (David and Pancharatna, 2009). These effects may be related to the mechanism of action of the PCM, evidenced in studies carried out by Cha et al. (2005, 2006) which have shown that cyclooxygenase inhibition induces defective formation of the vascular had, shortened intersomitic vessels, and causes growth arrest.

Zebrafish has been used the same way that it has been analyzed in most studies to assess the embryotoxic and teratogenic capacity of PCM. However, with the discovered findings in this study, we demonstrated that the common carp *C. carpio* can also be used as a good bioindicator for this type of tests because of its relevance as a species of economic and ecological interest in various Latin American regions (Gómez-Oliván et al., 2017).

In summary, PCM effects have shown outcomes at different levels. For example, the alteration of the chorion permeability, the ROS formation through its biotransformation including NAPQI so they have the ability to modify the biomolecules functions of common carp *C. carpio* embryos. This fish model is a good indicator of pollution from this non-steroidal anti-inflammatory.

5. Conclusions

PCM at environmentally relevant concentrations is capable of generating alterations to embryonic development and cause teratogenic effects in *C. carpio* embryos. The survival of 50% of embryos exposed to PCM was presented at the concentration of 1.29 μg/L. The effective concentration for malformations manifestation was 2.84 μg/L. PCM was embryolethal in accordance with the criteria established by Weigt et al. (2011). At concentrations of 2.0–3.5 μg/L, the most severe malformations occurred. Craniofacial malformations, skeletal disorders, pericardial edema and growth retardation were the most severe of all. The alterations to embryonic development and teratogenic effects contributed to the death of approximately 70% of the embryos in the highest concentrations tested. The environmentally realistic concentrations of PCM tested in this study altered the oxidative status in embryos of the common carp and at very low concentrations, the PCM has the ability to alter the *C.carpio* embryonic development of this economic interest specie.

CRediT authorship contribution statement

VMGN, MCRM, LMGO performed all the exposure experiments; LMGO and VMGN were involved in the conception; LMGO, HIF and VMGN were involved in the design and interpretation of the data and the writing of the manuscript with input from MGM and SGM.

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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