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Ibuprofen at environmentally relevant concentrations alters embryonic development, induces teratogenesis and oxidative stress in *Cyprinus carpio*

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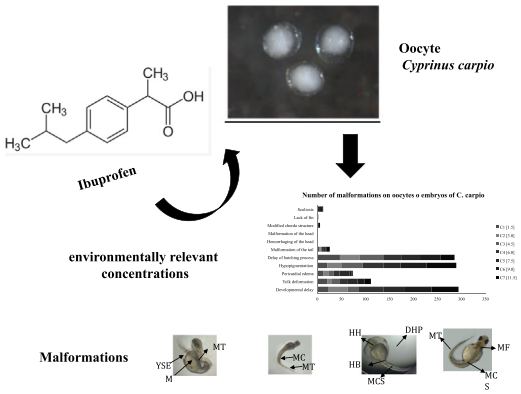
HIGHLIGHTS

- Ibuprofen malformations were determined using Kimmel and Hermsen method.
- Ibuprofen was capable of inducing alterations to embryonic development.
- The teratogenic index of the ibuprofen was 3, if >1 means teratogenic properties.
- Teratogenic effects were delayed hatching, hypopigmentation and pericardial edema.
- Ibuprofen at environmentally relevant concentrations is a dangerous agent for species of economic interest.

GRAPHICAL ABSTRACT



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ABSTRACT

Ibuprofen (IBU) is a non-steroidal anti-inflammatory (NSAIDs) that is used in various conditions. The prescriptions and the global consumption of this drug are very high and its annual production oscillates in millions of tons, this generates that the IBU is present in many waterbodies because it is discharged through the municipal, hospital and industrial effluents. For the above, the purpose of this work was to determine if IBU at environmentally relevant concentrations was capable of inducing alterations to embryonic development, teratogenic effects and oxidative stress in oocytes and embryos of *Cyprinus carpio*. Oocytes of common carp were exposed to IBU concentrations between 1.5 and 11.5 μg L^{-1} (environmentally relevant). LC_{50} and EC_{50} of malformations were determined to calculate the teratogenic index (TI). Also, main alterations to embryonic development and teratogenic effects were evaluated. Oxidative stress was evaluated by determining biomarkers of cellular oxidation and antioxidation using the same concentrations at 72 and 96 hpf in embryos of *Cyprinus carpio*. The results showed a LC_{50} of 4.17 μg L^{-1}, EC_{50} of 1.39 μg L^{-1} and TI of 3.0. The main embryonic development disorders and teratogenic effects were delayed hatching, hypopigmentation, pericardial edema, yolk deformation, and developmental

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delay. Biomarkers of cellular oxidation and antioxidants were increased with respect to the control in a concentration-dependent manner. The results of the study allow us to conclude that IBU at environmentally relevant concentrations is capable of inducing embryotoxicity and teratogenicity in a fish of commercial interest like *Cyprinus carpio*.

1. Introduction

Ibuprofen (IBU) is an analgesic and anti-inflammatory drug that is used for the treatment of painful conditions, accompanied by significant inflammation such as mild rheumatoid arthritis and musculoskeletal disorders (osteoarthritis, lumbago, bursitis, tendonitis, painful shoulder, sprains, among others) (Motov et al., 2019). This drug is used for the treatment of moderate pain in the postoperative period, in dental pain, primary dysmenorrhea and headache (Reed et al., 2018; Moore et al., 2019).

IBU is one of the most prescribed medications in the US, according to the annual Medical Expenditure Panel Survey conducted by the Agency for Healthcare Research and Quality (AHRQ), IBU is in the top 200 drugs of 2019 occupying the 35th place. According to this survey the prescriptions in 2016 were approximately 21,329,751 (Kane, 2018). A study conducted by Inotai et al. (2010), showed that in six countries of central and eastern Europe, diclofenac and IBU are the most widely used non-COX2 (cyclooxygenase 2) selective NSAIDs (non-steroidal anti-inflammatory). In Asia the most consumed NSAIDs in various countries are diclofenac, IBU, naproxen and acetylsalicylic acid (Delaney et al., 2011). The above demonstrates that IBU is widely consumed by the population in the world, which favors that this NSAID be released to aquatic environments through municipal, industrial and hospital effluents (Marchlewicz et al., 2015; Żur et al., 2018).

Several studies have detected the presence of IBU in influents and effluents from wastewater treatment plants (WWTP), surface water, drinking water, sludge and hospital effluent. The concentrations of IBU range from 0.001 to 75.8 μg L^{-1} in different effluents around the world (Table 1).

On the other hand, studies of the toxic effects of IBU in aquatic organisms are scarcer. Our research group has identified that IBU at concentration of 170 μg L^{-1} is capable of inducing oxidative stress in a freshwater amphipod *Hyalella azteca* (Gómez-Oliván et al., 2014b); at 18 μg L^{-1} has generated genotoxicity and oxidative stress in *Daphnia magna* (Gómez-Oliván et al., 2014a) and both oxidative stress and bioconcentration in different organs such as blood, liver, brain and gill of *Cyprinus carpio* at concentrations of 17.6 mg L^{-1} (Islas-Flores et al., 2014). Likewise, in other studies in the world the IBU has proven to be cytotoxic and genotoxic at concentrations of 0.2, 2 and 8 μg L^{-1} in freshwater bivalve *Dreissena polymorpha* (Parolini et al., 2011). In addition, IBU at 0.1 μg L^{-1} has been detected to affect fish reproduction, generate endocrine disruption with induction of vitellogenin in male fish, fewer broods per pair, and more eggs per brood in Japanese medaka *Oryzias latipes*, and freshwater cladocerans *D. magna* and *Moina macrocopa* (Han et al., 2010). Also the IBU at the concentration 2.5 μg L^{-1} has been shown to bioaccumulate, and cause alterations of immunological parameters, genotoxic effects, modulation of lipid metabolism and changes in cellular turn-over in *Mytilus galloprovincialis* (Mezzelani et al., 2018). Exposure of IBU significantly decreased the spontaneous movement by 25%, and reduced the free swimming distance, duration and speed under dark condition by 41%, 29% and 30%, at IBP concentrations of 5, 50 and 500 respectively on *Danio rerio* (Xia et al., 2017).

The background identified in our research group mentioned above and based on the fact that the IBU has been shown to generate oxidative stress (causing lipoperoxidation, increasing hydroperoxides content, carbonylating proteins and altering antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase), genotoxicity and cytotoxicity in species such as *D. magna*, *H. azteca* and *C. carpio* we are interested to identify if the IBU is capable of inducing embryotoxicity and teratogenesis in *C. carpio* embryos. This hypothesis is supported by several studies that have shown that the oxidative stress is related to alterations to embryonic development and teratogenicity in aquatic organisms (Combelles et al., 2009; Kupsco and Schlenk, 2015; Balbi et al., 2019; Pérez-Coyotl et al., 2019).

Therefore, the purpose of this work was to determine the LC_{50}, EC_{50} of malformations, teratogenic index, alterations to embryonic development, teratogenic effects and oxidative stress induced by IBU at environmentally relevant concentrations in oocytes and embryos of *C. carpio*.

2. Material and methods

2.1. Test specimens and oocyte harvest

The oocytes of *C. carpio* used in this study were provided by the Aquaculture Center of Tiacaque, in Jocotitlán, State of Mexico, which is the main producer of common and herbivorous carp in Mexico. The carps used in the study were adults of reproductive age with a size of 45 ± 5 cm and a weight of 3.8 ± 0.8 kg. *C. carpio* were contained in semi-rustic ponds with a water temperature between 19 and 23 °C. The carps were fed using a food enriched with protein and carbohydrates; in addition their diet was complemented with the natural flora of the boards ponds. In the case of males, the most viable were selected for spawning, analyzing their external characteristics. For females, those with a more bulky belly and the flushed genital area. Once the appropriate organisms were selected, they were transferred to the fertilization ponds where four females and eight males were placed to start the courtship. The fertilization process to obtain oocytes from *C. carpio* was carried out *in situ* and was by natural method. In the case of carp, fertilization was external, since while the male stimulated the females, they released eggs that synchronized with the expulsion of semen to fertilize them. Once fertilized, the eggs adhere to the nets that have been arranged around the ponds intended for fertilization and reproduction and also the bottom of the pond was covered with casuarina branches in order to deposit the oocytes obtained in them and facilitate its harvest. The oocytes harvested were classified by observation under the stereoscopic microscope and for the studies only those that were in the blastula stage (2 h post-fertilization) were used. Once the oocytes in blastula were selected, they were exposed to IBU environmentally relevant concentrations (that have been found in occurrence studies around the world considering influents and effluents from WWTP, surface water, drinking water, sludge and hospital effluent).

2.2. Exposure systems

The experiments were carried out following the guidelines established by the OECD in its (Test No. 236: Fish Embryo Acute Toxicity (FET) Test, 2013). Some adjustments were made to this guide considering the development of *C. carpio*, established by Luja-Mondragón et al. (2019). The environmentally relevant concentrations of IBU used in the study of embryotoxicity and teratogenicity were 1.5, 3, 4.5, 6, 7.5, 9 and 11.5 μg L^{-1}, in addition to an IBU free control system. These concentrations were selected considering studies of the occurrence and toxicity of IBU worldwide and previous studies

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

Country	Types of effluents and substrates in water bodies IBU concentrations (μg L^{-1})							References
	WWTP influent (min.–max.)	WWTP effluent (min.–max.)	Surface water (min.–max.)	Drinking water (min.–max.)	Sludge (min.–max.)	Groundwater (min.–max.)	Hospital influent (min.–max.)	
Canada	n.a.	0.077–2.051	0.007–0.790	n.a.	n.a.	n.a.	n.a.	Metcalfe et al., 2003a
Canada	14.2–75.8	0.3–24.6	n.a.	n.a.	n.a.	n.a.	n.a.	Metcalfe et al., 2003b
EE. UU	9.5–14.7	0.010–0.022	n.a.	n.a.	n.a.	n.a.	n.a.	Thomas and Foster, 2005
Germany	n.a.	n.a.	ND–2.383	n.a.	n.a.	n.a.	n.a.	Meyer et al., 2011
Greece	0.096–0.403	5×10^{-3}–0.262	n.a.	n.a.	n.a.	n.a.	0.254–0.574	Samaras et al., 2010
India	n.a.	n.a.	ND–0.200	n.a.	n.a.	n.a.	n.a.	Shanmugam et al., 2014
Iran	0.233–1.051	0.031–0.045	0.022–0.037	0.021–0.047	n.a.	n.a.	n.a.	Eslami et al., 2015
Latvia	n.a.	n.a.	0.004–0.018	n.a.	n.a.	n.a.	n.a.	Reinholds et al., 2017
Norway	n.a.	n.a.	0.001–0.005	0.001–0.009	n.a.	n.a.	n.a.	Reinholds et al., 2017
Portugal	n.a.	n.a.	ND–0.222	n.a.	n.a.	n.a.	n.a.	Lolić et al., 2015
South Africa	n.a.	n.a.	0.107–0.516	n.a.	n.a.	n.a.	n.a.	Archer et al., 2017
South Korea	n.a.	n.a.	ND–0.414	n.a.	n.a.	n.a.	n.a.	Kim et al., 2009
Spain	n.a.	0.433–2.633	2.358–11.891	0.029–0.571	n.a.	n.a.	n.a.	Heath et al., 2010
Sweden	n.a.	n.a.	n.a.	n.a.	0.115–0.129	n.a.	n.a.	Sagristà et al., 2010
Sweden	n.a.	n.a.	ND–0.818	n.a.	n.a.	n.a.	n.a.	Daneshvar et al., 2012
Switzerland	0.990–3.300	0.013–0.081	0.001–0.007	n.a.	n.a.	n.a.	n.a.	Buser et al., 1999
Taiwan	n.a.	n.a.	ND–4.350	n.a.	n.a.	n.a.	n.a.	Yu-Chen Lin et al., 2010

n.a. = not available; bql = below quantification limit, ND = not detected.

performed under controlled conditions using *C. carpio* and *D. magna* as bioindicators to assess oxidative stress (Gómez-Oliván et al., 2014c, 2014a; Heath et al., 2010; Islas-Flores et al., 2017, 2014; Kimmel et al., 1995; Kolpin et al., 2002; Metcalfe et al., 2011, 2003a; Meyer et al., 2011). To prepare the test systems, randomly selected *C. carpio* oocytes were placed in 24-well microplates. An oocyte was placed in each well to form batches of 20 oocytes for each concentration tested, the systems were replicated in quintuplicate. The microplates were placed in an incubator with oxygen supply for five days at 24 ± 1 °C, and with light control to keep them in light-dark cycles of 12:12 h. To evaluate if there was any embryonic alteration or teratogenic effect due to IBU, observations were made in the stereoscopic microscope at 12, 24, 48, 72 and 96 hpf, also photographs were taken using the Zeiss program for Windows.

2.2.1. Embryolethality test

The determination of lethal concentration 50 (LC_{50}) and effective concentration of malformations (EC_{50m}) was performed using the systems mentioned in Section 2.2. The determination of lethality parameters and teratogenic alterations was performed at 96 h. For the test, dead oocytes were considered when they were coagulated or no heartbeat was detected. After 96 hpf, the live, dead and malformed oocytes were counted and a maximum likelihood linear regression analysis was performed to calculate LC_{50} and EC_{50m} with their 95% confidence intervals ($p < 0.05$). For this purpose, US-EPA software ver 1.5 was used, using the Spearman-Karber method trimmed (Hamilton et al., 1977). The teratogenic index (TI) was also calculated using the ratio LC_{50}/EC_{50m}. If the TI was >1, IBU was considered as teratogenic and if it was <1 as embryolethal, according to criteria of Weigt et al. (2011).

2.2.2. Evaluation of embryotoxicity and teratogenic effects

The systems mentioned in Section 2.2 were used in the evaluation of embryonic development disorders and teratogenic effects. For this evaluation, live *C. carpio* oocytes and embryos were observed under a stereomicroscope at 12, 24, 48, 72 and 96 hpf. For this purpose the score established by Kimmel et al. (1995) and Hermsen et al. (2011) with modifications by Luja-Mondragón et al. (2019) was used. The determination of this score consisted of performing a quantitative and qualitative evaluation of each *C. carpio* embryo exposed to IBU and was compared with the control embryo, receiving points according to its development phase with respect to time. Embryonic development was assessed considering: 1) tail development, 2) formation of somites, 3) eye development, 4) movement, 5) blood circulation, 6) heartbeat, 7) head-body pigmentation, 8) pigmentation of the tail, 9) appearance of the pectoral fin, 10) mouth protuberance and 11) hatching.

With the alterations to the embryonic development and teratogenic effects identified, a frequency histogram of the main malformations induced by IBU exposure on oocytes and embryos of *C. carpio* was constructed using Statgraphics Centurion 18.

2.3. Evaluation of oxidative stress in Cyprinus carpio embryos

To evaluate cellular oxidation, the following biomarkers were used: hydroperoxide content (HPC), lipid peroxidation (LPX) and protein carbonyl content (PCC), while antioxidant defense was evaluated by measuring the activity of the enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Exposure systems were prepared using six liters of water added with 1.5, 3, 4.5, 6, 7.5, 9 and 11.5 μg L^{-1} of IBU, and IBU control system free was set up for each exposure

Table 2
Mortality and malformations rates by environmentally relevant concentrations of IBU exposure in *C. carpio* embryos.

IBU concentration μg L^{-1}	Mortality (%)	Malformations (%)
0	0	0
1.5	36	64
3	48	73
4.5	52	81
6	68	85
7.5	72	91
9	94	93
11.5	94 **LC50 = 4.17** **CI = [2.89–5.48]** **TI = 3.0**	100 **EC50 = 1.39** **CI = [0.71–2.01]**

TI = teratogenic index.

time. One gram of *C. carpio* oocytes was added to each system. The exposure times were 72 and 96. After the exposure period, 1 g of the organisms exposed in 1.5 mL of phosphate buffer (PBS, pH = 7.4) was homogenized. Samples were centrifuged at 15,000*g* × 15 min at 4 °C. The supernatant was used to determine HPC by Jiang et al. (1992) method, LPX by Buege and Aust (1978) method, protein carbonyl content (PCC) by Levine et al. (1994) method, and the activity of superoxide dismutase (SOD) by Misra and Fridovich (1972), catalase (CAT) by Radi et al. (1991), and glutathione peroxidase (GPx) by Stephensen et al. (2000) methods. Likewise, in order to normalize the results of the oxidative stress biomarkers evaluated and to express the results, the content of total proteins (PT) was determined by the Bradford (1976) method. The experiments were performed in fivefold.

2.4. Statistical analysis and test validity criteria

To evaluate sublethal toxicity assay results (oxidative stress biomarkers), after replication of data normality and homogeneity of variance (verified by Shapiro-Wilk and Levene's test), results were examined by one-way analysis of variance (ANOVA) followed by a Tukey-Kramer multiple comparisons test with 95% confidence limit to determine differences between means. SPSS v9 software (SPSS, Chicago, IL) was used.

For the dichotomous data (teratogenic and lethal effects) a log-logistic model was fitted. The concentration–response curves generated were required to determine EC50 (teratogenic effects) and LC50 (lethal effects) values. Based on LC50 and EC50 (teratogenic effects) values, a teratogenic index (TI) was calculated as the ratio LC50/EC50. These data were determined by PROBIT analysis (EPA Analysis Program v 1.5).

Embryotoxicity and teratogenic effects data were analyzed by Fisher's exact test, the frequency of abnormal oocytes or embryos was evaluated. Significance was accepted when $p < 0.05$, using SPSS v9 software (SPSS, Chicago, IL).

The validity criteria used in this study were principal two, the first was that the fertilization rate was ≥90%, and the second was that the test was considered valid if the control groups showed no >10% of lethal teratogenic effects at 96 hpf.

2.5. Ethical approval

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

3. Results

3.1. Embryolethality and teratogenicity data induced by IBU

The survival rate abruptly decreased, the mortality and malformation rates greatly increased starting with an ibuprofen concentration of 1.5 μg L^{-1}. These increases were concentration and time dependent (Table 2). This result indicates that the LC_{50} and LE_{50} values were around 4.17 μg L^{-1} and 1.39 μg L^{-1}, respectively, calculated with Stat graphics Centurion 18 software. Approximately 42 malformations in the lowest IBU concentration and 69 in the highest put the integrity of the oocytes at risk and delayed their hatching time. The highest tested IBU concentration (11.5 μg L^{-1}) induced death of almost all exposed embryos within 96 h after incubation in the solution generated the highest incidence of mortality (94%) and malformations (100%). The TI value for IBU was 2-times >1 (Table 2), demonstrating that IBU has potential developmental toxic and teratogenic effects during *Cyprinus* embryogenesis according to the criteria of Weigt et al. (2011).

The rate of live embryos decreased significantly (Probit analysis, $p \leq 0.05$) and mortality increased with increasing IBU concentrations (Fig. 1). Malformations increased significantly with increasing concentrations of IBU, however, at higher concentrations 7.5, 9.0 and 11. 5 μg L^{-1} there was a significant delay in hatching and death of embryos.

3.2. Main alterations to embryonic development and teratogenic effects induced by exposure to IBU

Various malformations were observed in embryos treated with IBU. Delay of the hatching process, hypopigmentation, pericardial edema, yolk deformation, developmental delay were the most common disorders observed after exposure to different concentrations of IBU (Fig. 2). However, tail malformations and scoliosis also occurred in both lower percentages and IBU concentrations.

The highest concentrations of IBU 9.0 and 11.5 μg L^{-1} showed approximately 56–57% developmental delay, 55–61% hypopigmentation and 30–42% delay in the hatching process. In contrast in the lower concentrations of IBU (1.5 and 3.0 μg L^{-1}), a higher incidence of malformations such as pericardial edema, tail deformation and delayed somite formation were observed (Table 3).

3.3. Embryonic development score at different times and concentrations

C. carpio embryos exhibited diverse and specific morphological abnormalities after exposure to IBU. The incidence of abnormal embryos (fraction of embryos with some teratogenic effect) and delayed embryos (fraction of embryos with some delay as measured by the

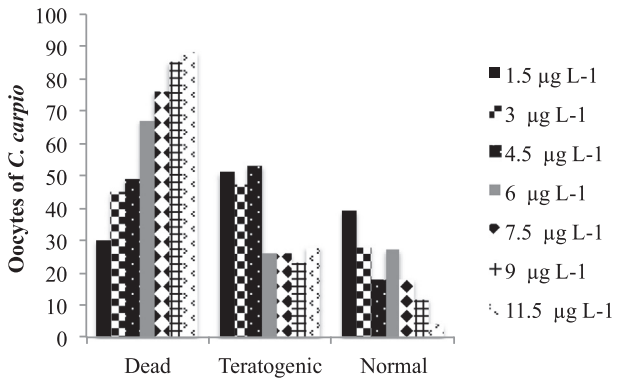


Fig. 1. Survival, malformation and teratogenic embryos exposed to IBU concentrations.

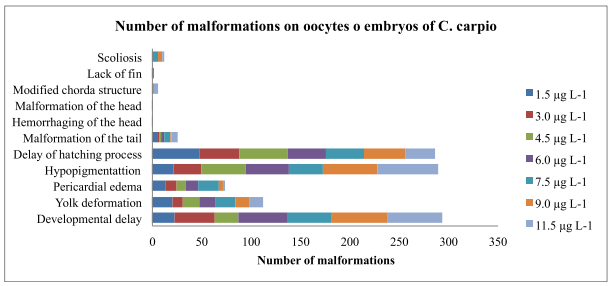


Fig. 2. Main malformations induced by exposure to IBU concentrations in *C. carpio* embryos.

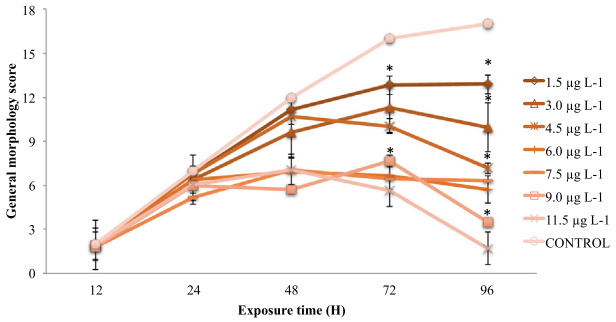


Fig. 3. Concentration-response curves of IBU in *C. carpio* embryos.

morphological score applied) was dependent on the concentration of IBU at all times. In Fig. 3, a clear change was observed between the concentration-response curves with respect to the control (Fisher test, $p \leq 0.05$), suggesting that there is a progressive effect on development at high IBU concentrations. All groups of embryos exposed to IBU concentrations decreased statistically significantly with respect to the control group (Fisher test, $p \leq 0.05$).

Control images represent the normal development of the oocyte, until it hatches at the different hpf. BU exposures in low concentrations (1.5 and 3.0 µg L^{-1}) resulted in malformations of tail, yolk deformation, modified chorda structure and yolk edema (Fig. 4). The most common disorders observed in high IBU concentration (11.5 µg L^{-1}) were severe cases of scoliosis, alterations of the notochord, pericardial edema, and malformations of tail. The highest IBU concentrations in the longest exposure times resulted in severe malformations that put the life of common carp embryos at risk, presenting a high percentage of mortality (94%). The results of Table 4 clearly show how very low concentrations of IBU are capable of generating embryonic alterations and teratogenic effects in *C. carpio* embryos.

3.4. Oxidative stress biomarkers

Table 3 shows the biomarkers of cell oxidation and antioxidation at 72 and 96 hpf. As can be seen, the values of all biomarkers were increased with respect to the control group and the exposure time in a statistically significant manner, and concentration dependent in *C. carpio* embryos.

Increases of up to approximately 138% were observed for the hydroperoxides content, 98.4% for the LPX and 256.1% for carbonylated proteins with respect to the control groups ($p \leq 0.05$). In the case of antioxidant activity, increases of up to 434.8, 97.8 and 447.1 were observed in the activities of the SOD, CAT and GPx enzymes relative to the control group ($p \leq 0.05$). These increases were time and concentration dependent in all cases.

4. Discussion

IBU is the third non-steroidal anti-inflammatory drug most used in the world. Its growing consumption reflects the ubiquitous occurrence of this pharmaceutical in the aquatic environment (Brun et al., 2006). This drug has been constantly detected in many water bodies, rivers and wastewaters and the concentrations in which it has been detected are in the range of ng L^{-1}–µg L^{-1} (Buser et al., 1999; Tran et al., 2014). This is a global problem concern as IBU tends to accumulate and produce toxic effects in diverse aquatic organisms (Mezzelani et al., 2018).

IBU has been associated with the alteration oxidative stress biomarkers in multiple species. Gonzalez-Rey and Bebianno (2011) demonstrated the breakdown of the redox defense system and the IBU prooxidant activity in mussels exposed to environmental concentrations of this pollutant. Their results agree with those reported by Bartoskova et al. (2013); Islas-Flores et al. (2014); Stancova et al. (2017), who also demonstrated this pharmaceutical induced oxidative stress in zebrafish, common carp and tench, respectively.

Alterations in the redox system have been associated with alterations in embryonic development and teratogenic effects in aquatic organisms (Balbi et al., 2019; Pérez-Coyotl et al., 2019).

For the above, in this investigation, we decided to identify the main alterations to embryonic development, teratogenic effects and oxidative stress due to exposure to IBU in *C. carpio* oocytes and embryos, motivated because in previous studies performed in the lab we have identified that IBU has been able to generate oxidative stress in juvenile stages of *C. carpio* in organs such as blood, gills, liver and brain (Islas-Flores et al., 2017, 2014). Likewise, it has generated this same response in other aquatic species such as *Daphnia magna* and *Hyalella azteca* (Gómez-Oliván et al., 2014a, 2014b).

The phenomenon of oxidative stress occurs when the rate of generation of reactive oxygen species (ROS) at the cellular level exceeds its elimination from organisms, resulting in deleterious effects in organisms such as the oxidation of lipids, proteins and DNA (Martínez-Álvarez et al.,

Table 3
Percentages of IBU-induced malformations at environmentally relevant concentrations.

Type of malformation	Ibuprofen concentration (µg L^{-1})						
	1.5 µg L^{-1}	3.0 µg L^{-1}	4.5 µg L^{-1}	6.0 µg L^{-1}	7.5 µg L^{-1}	9.0 µg L^{-1}	11.5 µg L^{-1}
Developmental delay	22	41	24	50	44	57	56
Yolk deformation	20	11	16	17	20	15	13
Pericardial edema	13	11	10	12	21	4	3
Hypopigmentation	21	29	44	44	35	55	61
Delay of hatching process	47	41	49	39	38	42	30
Malformation of the tail	5	2	2	3	6	1	7
Hemorrhaging of the head	0	0	0	0	1	0	0
Malformation of the head	0	0	0	0	1	0	0
Modified chorda structure	0	0	0	0	0	2	4
Lack of fin	0	1	0	0	1	0	0
Scoliosis	0	0	0	0	6	4	2

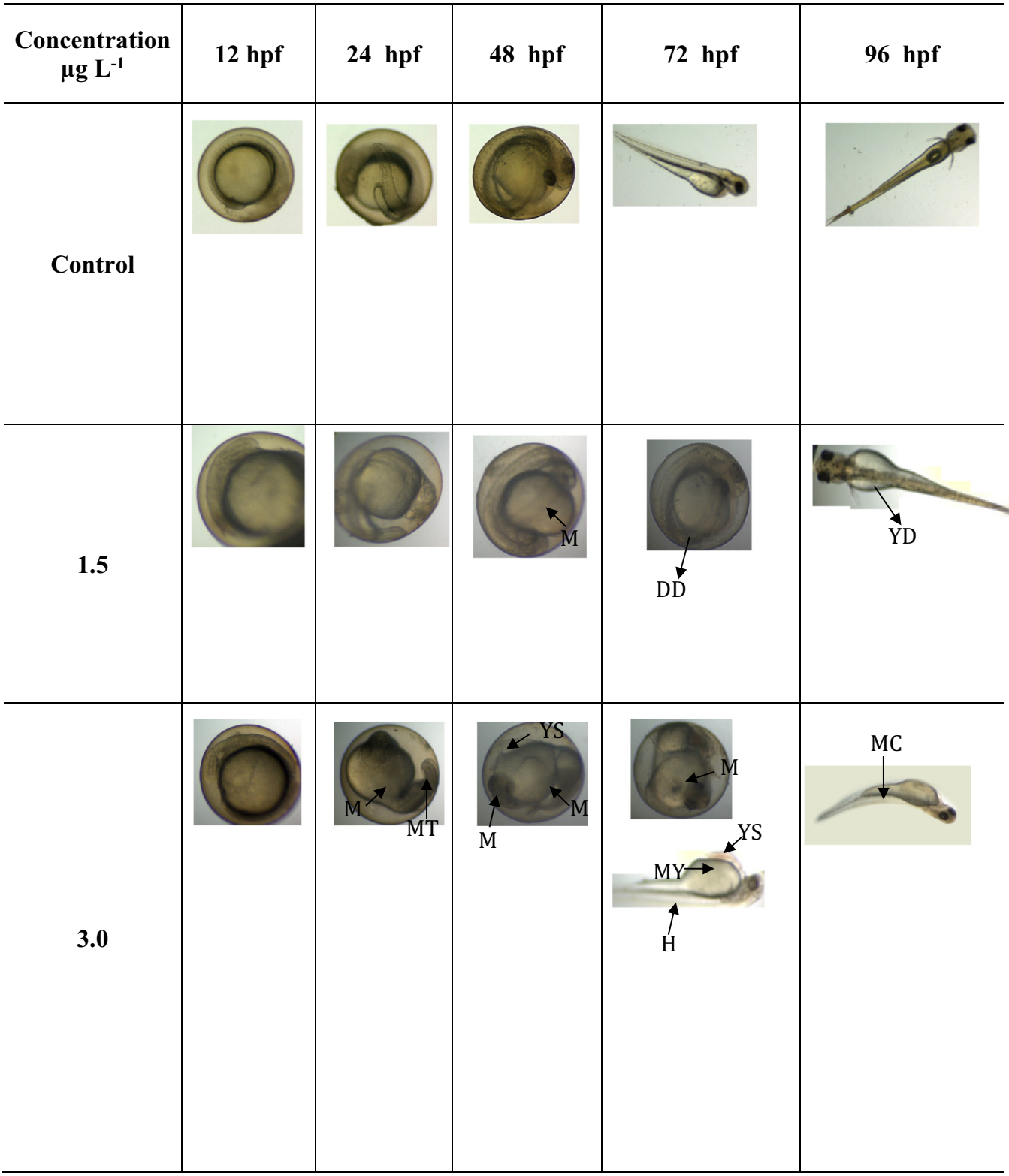


Fig. 4. Effects of IBU exposure on morphological feature in *C. carpio* embryos.

2005). In addition, apoptosis, delays in embryonic development and teratogenic effects in organisms can be generated (Pašková et al., 2011). There are different mechanisms identified by which the IBU can generate oxidative stress. For example the IBU that is a derivative of propionic acid has the ability to generate ROS in the presence of light (Husain et al., 2015). Likewise, NSAIDs such as IBU have the ability to decouple or inhibit oxidative phosphorylation generating ROS (Will et al., 2019). Another mechanism through which the IBU generate ROS is through its biotransformation by the CYP, producing 2 and 3 hydroxy derivatives (Gao et al., 2018; Islas-Flores et al., 2014). In this process the radical

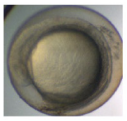
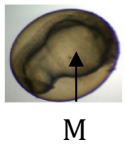
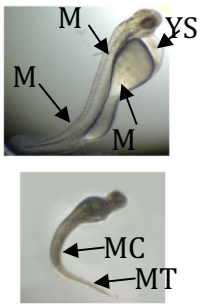
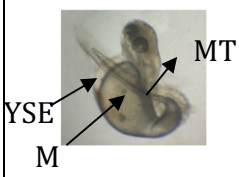
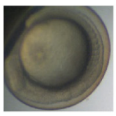
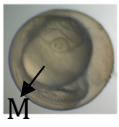
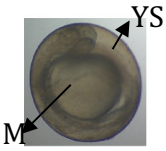
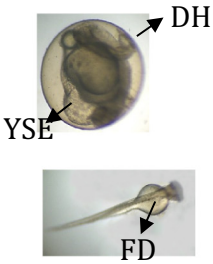
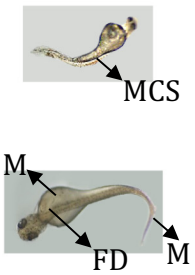
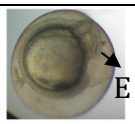
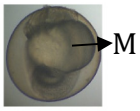
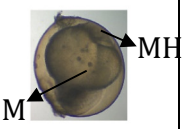
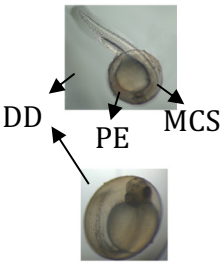
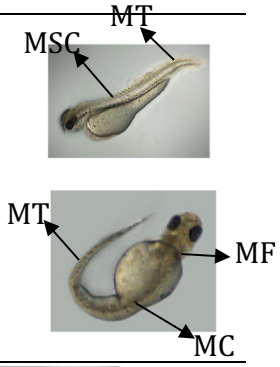
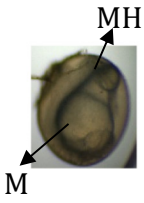
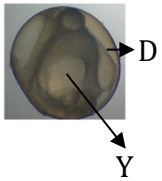
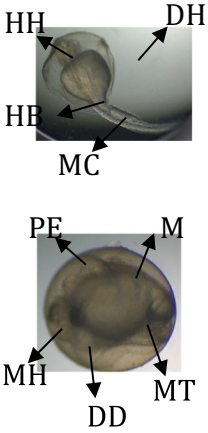
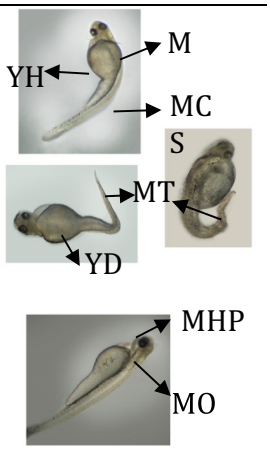
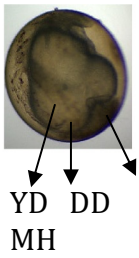
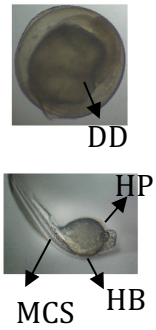
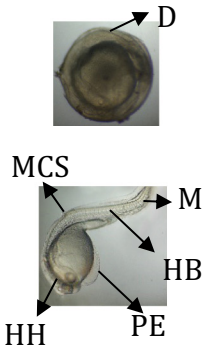
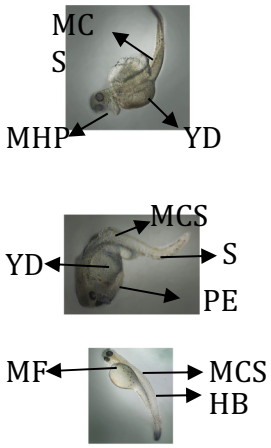
Concentration µg L^{-1}	12 hpf	24 hpf	48 hpf	72 hpf	96 hpf
4.5					
6.0					
7.5					
9.0					

Fig. 4 (*continued*).

anion superoxide and hydrogen peroxide are formed and can be responsible for generating oxidative stress (Doi et al., 2002). The biotransformation of IBU by CYP2C9 generates ROS such as the hydroxyl radical (OH•), and the superoxide anion (O_2•) (Uno et al., 2012).

It is important to highlight that the oxidative stress has been associated with the pathogenesis of various abnormalities; within these, skeletal malformations and cardiovascular defects (Kovacic and Somanathan, 2014). ROS have an important role in embryonic

Concentration µg L^{-1}	12 hpf	24 hpf	48 hpf	72 hpf	96 hpf
11.5					

DHP= delay in the hatching process**; H =** hypopigmentation**; HH** = hemorrhaging in the head; **HT** = hemorrhaging in the tail; **HY =** hemorrhaging in the yolk**; M** = miscellaneous; **MS**= severe malformations; **MCS** = modified chorda structure; **MH** = malformation of the head; **MHE** = malformation of the heart; **MT** = malformation of tail; **PE =** pericardial edema**; S** = scoliosis; **YD** = yolk deformation; **YSE** = yolk edema

Fig. 4 (*continued*).

development, since its can be to alter embryonic signal transduction pathways (Dennery, 2007; Wells et al., 2005). These facts, we were able to corroborate in this study, since by exposing *C. carpio* embryos to IBU concentrations between 1.5 and 11.5 µg L^{-1}, we identified alterations in cellular oxidation and antioxidation biomarkers, time and concentration dependent.

The early stages of embryonic development in various aquatic organisms there is a high susceptibility to oxidative damage. This occurs

Table 4
Biomarkers of cellular oxidation and antioxidation in *C. carpio* embryos at 72 and 96 h.

Concentration µg L^{-1}	HPC nM of CHP mg PT^{-1}		LPX mM MDA mg PT^{-1}		PCC µM C=O mg PT^{-1}		SOD IU SOD mg PT^{-1}		CAT µM H_2O_2		GPx mM NADPH mg PT^{-1}	
	72 hpf	96 hpf	72 hpf	96 hpf	72 hpf	96 hpf	72 hpf	96 hpf	72 hpf	96 hpf	72 hpf	96 hpf
Control (0)	0.60 ± 0.01	0.62 ± 0.01	65.3 ± 0.8	67.4 ± 0.8	0.41 ± 0.01	0.45 ± 0.03	0.23 ± 0.01	0.27 ± 0.3	92 ± 0.8	95 ± 01.3	0.017 ± 0.003*	0.019 ± 0.005*
1.5	0.71 ± 0.03*	0.73 ± 0.01*	71.3 ± 0.7*	74.7 ± 0.9*	0.52 ± 0.07*	0.59 ± 005*	0.32 ± 0.07*	0.37 ± 0.04*	102 ± 0.9*	107 ± 0.6*	0.023 ± 0.005*	0.027 ± 0.006*
3.0	0.82 ± 0.02*	0.84 ± 0.02*	81.6 ± 0.7*	84.8 ± 0.6*	0.63 ± 0.02*	0.71 ± 0.03*	0.45 ± 0.05*	0.49 ± 0.03*	113 ± 1.1*	122 ± 1.7*	0.031 ± 0.007*	0.039 ± 0.005*
4.5	0.95 ± 0.03*	0.97 ± 0.02*	93.5 ± 0.5*	97.5 ± 0.8*	0.81 ± 0.05*	0.86 ± 0.07*	0.53 ± 0.04*	0.58 ± 0.05*	132 ± 0.7*	139 ± 0.5*	0.045 ± 0.005*	0.049 ± 0.003*
6.0	1.03 ± 0.04*	1.06 ± 0.05*	101.6 ± 1.4*	106.3 ± 1.6*	0.93 ± 0.07*	0.95 ± 0.05*	0.66 ± 0.05*	0.72 ± 0.05*	141 ± 1.3*	148 ± 1.7*	0.056 ± 0.004*	0.061 ± 0.007*
7.5	1.23 ± 0.03*	1.25 ± 0.06*	109.8 ± 1.2*	111.7 ± 1.5*	0.98 ± 0.08*	1.03 ± 0.06*	0.84 ± 0.07*	0.96 ± 0.05*	153 ± 0.9*	161 ± 0.5*	0.073 ± 0.005*	0.081 ± 0.004*
9.0	1.32 ± 0.03*	1.35 ± 0.05*	118.6 ± 1.3*	122.5 ± 1.1*	1.15 ± 0.07*	1.23 ± 0.05*	1.03 ± 0.05*	1.15 ± 0.07*	167 ± 0.9*	172 ± 0.6*	0.088 ± 0.005*	0.091 ± 0.004*
11.	1.43 ± 0.02*	1.47 ± 0.04*	125.6 ± 0.7*	133.7 ± 1.1*	1.46 ± 0.05*	1.52 ± 0.07*	1.23 ± 0.06*	1.32 ± 0.05*	182 ± 0.7*	193 ± 1.1*	0.093 ± 0.003*	0.098 ± 0.005*

CHP = cumene hydroperoxide; MDA = malondialdehyde; C=O = reactive carbonyls.
Values are the mean of five replicates ± SEM.
* Significantly different ($p < 0.05$) from the control group. ANOVA and Tukey-Kramer.

as a result of the aerobic metabolism overload due to the high energy demand that occurs in the growth process. In addition to the conditions that lead to high production of free radicals, high iron levels not bound to proteins (NPBI) and lack of maturity of antioxidant systems (Mohd Zanuri et al., 2017; Petitjean et al., 2019).

As can be seen in the embryolethality study, 3 μg L^{-1} of IBU resulted in deaths of approximately 50% of *C carpio* larvae. These findings could be related to the IBU mechanism of action in mammals that is to inhibit the enzymes cyclooxygenase (COX-1 and COX-2) (Bittencourt et al., 2019), and that it has been identified that in fish it is similar (Cha et al., 2006). In particular, COX-1 has been linked to a cessation of fish growth and it is also important to consider that prostaglandins that are inhibited by NSAIDs exposure are indispensable in both gastrulation and segmentation process (Cha et al., 2006, 2005). A study performed by David and Pancharatna (2009a, 2009b), was observed that IBU at a concentration of 5 μg L^{-1} generated approximately 30% of the death of larvae in *Danio rerio*. So, we could infer that *C. carpio* larvae are more sensitive to this drug than zebra fish.

Most effects of IBU have been identified in adult organisms rather than in the initial stages of life. For example, the exposure of adult organisms of *Danio rerio* to IBU at 1 μg L^{-1} induced alterations in reproduction, decreased the number of spawned eggs, at 10 μg L^{-1} exposure as well decreased hatching rate of the progeny. The IBU is able to increase in the mortality, decline maturation of sperm and gametogenesis, and produce developmental abnormalities, the most observed abnormalities due IBU exposure were: cardiac edema and spinal malformations, of exposed embryos was also manifested (Ji et al., 2013).

The main embryonic development alterations identified in this study were developmental delay and hatching process delay. These alterations could be due to the inhibition of important enzymes in the hatching process such as chorionase (Haendel et al., 2004), by the presence of ROS, or because of the oxidative damage that ROS generate in the chorion of *C carpio* oocyte. In addition, as in the hatching process the oxygen requirements are greater in the oocytes, the imbalance in the redox status of the carp can cause developmental alterations (Pašková et al., 2011).

The findings identified in this study can be confirmed with the results obtained by Flippin et al. (2007), who demonstrated that by exposures at concentrations of 1–100 μg L^{-1} of IBU, there was a decrease in the number of spawning events per week of Japanese medaka, *Oryzias latipes*. Also the studies carried out by Han et al. (2010) showed that exposure to IBU in a concentration of 100 μg L^{-1} delayed the hatching of the eggs of *Oryzias latipes*. In our study we found delayed hatching process in the highest concentrations of IBU (11.5 μg L^{-1}).

The results are consistent also with other authors who have demonstrated the IBU effects such as developmental delay, cardiac anomalies, pectoral fin malformation, decreased hatching rate and growth, spinal curvature, and behavioral alterations in *Danio rerio* at concentrations > 10 μg L^{-1} (David and Pancharatna, 2009a, 2009b).

Previous studies conducted by our research group have shown that the IBU generates oxidative stress in sentinel species such as *D. magna*, *H. azteca* and *C. carpio*, as well as, genotoxic and cytotoxic effects in juveniles of *C. carpio*. The results of this study also allowed us to identify that the IBU is capable of generating alterations to embryonic development, teratogenic effects and oxidative stress in embryos of *C. carpio*. These data allow us to identify that IBU is a dangerous substance for common carp in very low concentrations.

5. Conclusions

IBU at environmentally relevant concentrations 1.5–11.5 μg L^{-1} is capable of inducing alterations to embryonic development and teratogenic effects in oocytes and embryos of the common carp *C. carpio*. The teratogenic index of the IBU was 3.0 and the most common embryonic development disorders and teratogenic effects were delay of the hatching process, hypopigmentation, pericardial edema, yolk deformation, developmental delay. With the observed effects we can conclude that IBU at environmentally relevant concentrations in water is a dangerous agent for species of economic interest such as *C. carpio*.

Declaration of competing interest

The authors declare they have no actual or potential competing financial interests.

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