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Developmental alterations, teratogenic effects, and oxidative disruption induced by ibuprofen, aluminum, and their binary mixture on *Danio rerio*☆

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ABSTRACT

Several studies highlighted the ubiquitous presence of ibuprofen and aluminum in the aquatic environment around the world and demonstrated their potential to induce embryotoxic and teratogenic defects on aquatic species individually. Although studies that evaluate developmental alterations induced by mixtures of these pollutants are scarce; and, since environmental contamination presented in the form of a mixture of toxicants with different chemical properties and toxicity mechanisms capable of generating interactions; the objective of this study was to evaluate the developmental defects, teratogenic alterations, and oxidative stress induced by individual forms and the mixture of ibuprofen (IBU) and aluminum (Al) on zebrafish embryos. Oocytes exposed to environmentally relevant concentrations of IBU (0.1–20 μg L-1) and Al (0.01–8 mg L-1) and one binary mixture. The LC50 and EC50 were obtained to calculate the teratogenic index (TI). The IBU LC50, EC50, and TI were 8.06 μg L-1, 2.85 μg L-1 and 2.82. In contrast, Al LC50 was 5.0 mg L-1with an EC50 of 3.58 mg L-1 and TI of 1.39. The main alterations observed for individual compounds were hatching alterations, head malformation, skeletal deformities, hypopigmentation, pericardial edema, and heart rate impairment. The mixture also showed significant delays to embryonic development.

Moreover, oxidative stress biomarkers of cellular oxidation and antioxidant defenses at 72 and 96 hpf significantly increased. Results show that environmentally relevant concentrations of ibuprofen (IBU), aluminum (Al), and their mixture promote a series of developmental defects, teratogenic effects, and oxidative disruption on *D. rerio* embryos, and the interaction of both substances altered the response. In conclusion, morphological and biochemical tests are suitable tools for assessing the health risk of aquatic wildlife by exposure to individual and mixed pollutants in freshwater bodies.

1. Introduction

Aquatic discharges of chemical contaminants have become one of the most relevant issues of concern over the past few years. The constant release of natural and anthropogenic substances into the aquatic environment even at trace levels is known to promote noxious effects not only in the aquatic system itself but also in the living organisms due to the use, disposal, fate, and incomplete removal of pollutants at WWTPs (Gorito et al., 2017; Radović et al., 2015).

Aluminum (Al) is ubiquitous in the Earth's lithosphere; it exhibits a complex biogeochemical cycle and wide distribution in natural environments (García-Medina et al., 2010; Schroniltgen et al., 2007). Also, this metal can be released from natural sources such as geological processes, atmospheric depositions, and acidification of aquatic systems or,

Abbreviations: Al, aluminum; CAT, catalase; CYP450, cytochrome P450; EC50, effective concentration of malformations 50; GPX, glutathione peroxidase; HPX, hydroperoxides; IBU, ibuprofen; LC50, lethal concentration 50; LPO, lipid peroxidation; MOA, mode of action; NSAIDs, non-steroidal anti-inflammatory drugs; OS, oxidative stress; PCC, protein carbonyl content; PGs, prostaglandins; ROS, reactive oxygen species; RNS, reactive nitrogen species; SOD, superoxide dismutase; TI, teratogenic index; TP, transformation products; WWTPs, wastewater treatment plants.

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to a greater extent, anthropogenic activities, mainly agriculture, manufacturing industry, mining, fossil fuel combustion, and WWTPs processes (Authman, 2015; Bondy, 2010; EPA, 2017; Fernández-Dávila et al., 2012; García-Medina et al., 2010; Lanctôt et al., 2017; Michelena et al., 2016). Al concentrations have been reported between 6.0 and 24.45 mg L^{-1} in Madin reservoir in Mexico (González-González et al., 2014), while in China, Li et al. (2016) reported concentrations up to 818 μg L^{-1} in Chaohu Lake surface waters. In recent years, Reutova et al. (2018) identified concentrations between 65 and 625 μg L^{-1} of Al in different rivers sampled in the central Caucasus. Regarding its toxic effects, in mammals, Al exposure is believed to be a significant agent in the pathology of neurodegenerative disorders such as Alzheimer's and Parkinson's diseases (Mathiyazahan et al., 2015), amyotrophic lateral sclerosis (McLachlan et al., 2019), and senile dementia (Reutova et al., 2018). While, in aquatic organisms, environmental exposure has been related to the induction of oxidative stress (Fernández-Dávila et al., 2012), geno- and cytotoxicity (Gómez-Oliván et al., 2017), embryotoxicity and teratogenic malformations (Kovrižnych et al., 2013; Monaco et al., 2017), behavioral and morphological disturbances (Grassie et al., 2013; Griffitt et al., 2008) and neurotoxic effects (Monaco et al., 2017).

On the other hand, pharmaceutical compounds and their transformation products (TPs) are considered pollutants of emerging concern (Laws et al., 2011). Between these, ibuprofen (IBU) is an over-the-counter pharmaceutical that acts as a non-selective inhibitor of the cyclooxygenase enzyme system, in particular, COX-1 and COX-2, implicated in the transformation of arachidonic acid to prostaglandins and thromboxane (Bjarnason et al., 2018; Fitzpatrick, 2005; Jamil et al., 2016; Santos et al., 2010); and has been widely detected in aquatic environments worldwide. In South Africa, Madikizela and Chimuka (2017) reported the occurrence of IBU residues in wastewater (5.1 up to 72 μg L^{-1}) and surface samples (4.8 up to 11 μg L^{-1}); while González-Alonso et al. (2017) measured concentrations of 0.037–10.05 μg L^{-1} in natural and wastewater discharges in the Antarctic Peninsula. Furthermore, IBU has shown to induce biochemical, morphological, and behavioral disturbances to aquatic species at trace levels (Bartoskova et al., 2013; Gómez-Oliván et al., 2014; Grzesiuk et al., 2020; Islas-Flores et al., 2017; Senger et al., 2011; Xia et al., 2016).

It is a fact that the pollutants found in the aquatic environment are usually presented as mixtures, not as an individual; therefore, studies on risk assessment for aquatic life must consider this complex exposure situation. The evaluation of complex mixtures of various substances has been the subject of concern of various researchers for years who have carried out studies on this topic using concepts initially developed by pharmacologists in the first half of the 20th century (Loewe and Muischnek, 1926; Bliss, 1939; Cleuvers, 2003, 2004; Gauthier et al., 2014; Lawrence et al., 2012). However, and in contrast to a large amount of analytical data on environmental toxicology, studies evaluating possible ecotoxicological effects are scarce, and only very few contain data on the toxicity of mixtures (Cleuvers, 2003), which are essential since depending on their chemical properties, toxicity mechanisms, and interaction potential they can have drastically different effects ex and in vivo (Gauthier et al., 2014).

As mentioned before, due to the different sources and routes of entry into the environment, specifically metals and pharmaceuticals, these mixtures in effluents are common; however, information on their interaction and toxic effects is scarce. For example, studies carried out by González-González et al. (2014), Martínez-vieyra et al. (2017) and Pérez-Alvarez et al. (2018) have found the combined presence of heavy metals (Fe, Al, Hg, As, Cu, Ni, Cr, Cd, Pb) and NSAIDs (DCF, IBP. NPX, PAR) in different water samples of Mexico at concentrations above the maximum levels permitted for the protection of aquatic life with noxious consequences to the biota; as the induction of several toxicity mechanisms at early stages of development (Escobar-Huerfano et al., 2020; Luja-Mondragón et al., 2019; SanJuan-Reyes et al., 2020); Gutiérrez--Noya et al. (2020) found that IBU at environmentally relevant concentrations (1.5–11.5 μg L^{-1}) induced major embryonic developmental disorders and teratogenicity on *C. carpio* oocytes; while, Tenorio-Chávez et al. (2020) evaluated the lethality and malformations present in zebrafish embryos after exposure to a hospital effluent containing several pharmaceuticals and heavy metals, concluding that these pollutants can promote severe alterations to embryonic development and teratogenic effects.

As aquatic ecosystems are frequently polluted with a variety of substances with diverse physicochemical properties and toxicity mechanisms, Zebrafish (*Danio rerio*) is considered a suitable animal model for the assessment of environmental risk associated with aquatic pollutants and their potential interactions in non-target organisms (Scholz et al., 2008) due to numerous advantages, including small size, rate of fecundity, the optical clarity of embryos, and complete genome sequence (Hill et al., 2005; Kari et al., 2007; Spitsbergen and Kent, 2003).

Due to the above and the scarce information reported on mixtures, the present study focuses on evaluating embryonic development alterations, teratogenic effects, and oxidative stress induced by IBU, Al, and the binary mixture on *Danio rerio* embryos.

2. Materials and methods

2.1. Test substances

Aluminum stock solutions (1 g L^{-1}) were prepared with anhydrous aluminum chloride ($AlCl_3$, ReagentPlus® CAS Number 7446-70-0; >99.9% purity) in deionized water (pH 6.0 ± 0.3 and 3 h aging). Stock solutions were not filtered before exposure (EPA, 2017).

IBU stock solutions (1 g L^{-1}) were prepared with Ibuprofen (CAS number 15687-27-1, ≥98% purity). Stock solution were prepared with deionized water. Unless otherwise indicated, reagents were provided by Sigma-Aldrich, St Louis.

2.2. FET assay

Exposure assay was carried out following the OECD Guidelines for the Testing Chemicals: Fish Embryo Toxicity (FET) Test (OECD, 2013).

2.2.1. Parent fish procurement

A breeding stock of unexposed healthy wild-type zebrafish specimens in sexual maturity (0.89 ± 0.3 g weight and 3.52 ± 0.5 cm length) was used for egg production. Fish were sexed and kept in 50-L vessels with dechlorinated tap water at 26 ± 2 °C, pH ranging between 7.2 and 7.6, oxygen saturation above 60%, and natural photoperiods of 12 h dark and 12 h light. Specimens were fed with dry flake food three times a day according to the methodologies followed by Ferdin and Halili (2017); Senger et al. (2011), and recommendations cited in OECD (2013). Two weeks before spawning, specimens were exclusively fed with *Artemia* sp. (OECD, 2013).

2.2.2. Oocyte harvest

Females and males (2:1) were placed into three individual 10-L spawning vessels with dechlorinated tap water reconstituted with commercial salts (1 mL/L) at 27 ± 1 °C. Mesh boxes were placed inside the vessels to prevent predation. Harvested oocytes were randomly selected and observed under a stereoscopic microscope (Zeiss Stemi 305) to separate viable and non-viable eggs. Only fertilized embryos at the blastula stage (4–6 hpf) were used (OECD, 2013).

2.2.3. Toxicity tests of isolated compounds

Viable oocytes were exposed to 0.1, 0.5, 1, 3, 5, 7, 9, 11, 20 μg L^{-1} and 0.01, 0.05, 0.1, 1, 2, 4, 6, 8 mg L^{-1} of IBP and Al, respectively. The selection of concentrations used in this study was based on the results of studies performed by Archer et al. (2017); Ginebreda et al. (2010); González-González et al. (2014); Madikizela and Chimuka (2017). Batches of 20 eggs were used for each concentration and free control

(OECD, 2013). Quintuplicates were performed. Embryos were placed in plates with 24 wells filled with 2 mL of reconstituted medium containing different concentrations of IBU and Al and maintained in an incubator for 96 h at 27 ± 0.5 °C. Observations were made with the stereomicroscope at 12, 24, 48, 72, and 96 hpf. The determination of embryo-lethality and teratogenic effects was carried out at 96 hpf. Lethality was considered when oocytes were coagulated or with a lack of heartbeat.

2.2.3.1. Determination of LC50, EC50, and teratogenic index of IBU and Al. After 96 hpf, the live, dead, and total malformed embryos were quantified, and a maximum likelihood linear regression analysis was used to calculate the Lethal Concentration 50 and Effective Concentration 50 values ($p<0.05$). The Spearman- Karber method (US-EPA software ver 1.5) was performed. The Teratogenic Index (TI) of both ibuprofen and aluminum was calculated according to the quotient between LC50/EC50 (Weigt et al., 2011).

2.2.4. Toxicity tests of the binary mixture

To evaluate the lethality and teratogenic alterations induced by the mixture of IBU and Al, oocytes were exposed to nominal concentrations of both toxicants equal to the EC50 values of isolated forms (2.85 μg L^{-1} of IBU and 3.58 mg L^{-1} of Al). Afterward, the methodology of maintenance and analysis was performed as indicated in section 2.2.3.

2.2.5. Evaluation of embryonic development of organisms exposed to isolated forms and binary mixture

Systems mentioned in sections 2.2.3 and 2.2.4 were used for the qualitative and quantitative evaluation of developmental features and teratogenic effects of embryos according to their visible morphology at 12, 24, 48, 72, and 96 hpf. Structural abnormalities and delayed development were contemplated as the main teratogenic endpoints. All embryos were compared with reference embryos presented by Kimmel et al. (1995). Endpoints such as eye, and tail development, somite formation, heartbeat, blood circulation, movement, head-body, and tail pigmentation, pectoral fin appearance, mouth protuberance, and hatching rate were evaluated according to the score established by Hermsen et al. (2011).

2.2.6. Malformations and teratogenic effects analysis of organisms exposed to isolated forms and binary mixtures

Quantitation of malformations and teratogenic alterations were recorded in a database after 12, 24, 48, 72, and 96 hpf. In search of any visible malformation such as pericardial and ocular edema, head, otolith, tail and heart malformation, modified notochord structure (scoliosis), and delay in the hatching process (Hermsen et al., 2011), all organisms were observed under the stereomicroscope and photographed for later analysis. After examination, biological samples were disposed of following institutional standards.

2.3. Heart rate assessment

Ten larvae per concentration group (72 hpf) were placed in petri dishes containing 3% of methylcellulose according to the methodology followed by Antkiewicz et al. (2005) and heart images were recorded with a Zeiss Axiocam 5s camera. Ventricular and atrial heartbeats were counted in 15 s periods and then calculating the beats per minute. Triplicates were performed. Results were compared to the control group placed under identical conditions.

2.4. Oxidative stress biomarkers

The following biomarkers were evaluated: hydroperoxide content (HPX), lipid peroxidation (LPO), protein carbonyl (PCC), and antioxidant activity of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). After exposure (72 and 96 hpf), *D. rerio* oocytes were weighed, placed in vials containing PBS (pH = 7.4) and homogenized. Samples were centrifuged at 15,000 g x for 20 min at 4 ± 0.5 C° and stored. The supernatant was used to determine HPX according to the method proposed by Jiang et al. (1992); PCC by Levine et al. (1994); LPX with the TBARs method (Buege and Aust, 1978) as well as SOD according to the method proposed by MARKLUND and MARKLUND (1974) and modified by Li (2012); CAT using the methodology by Radi et al. (1991) and GPX as specified by Gonzler (1984) and modified by García-Medina et al. (2013). Also, the content of total proteins (TP) was determined according to Bradford (1976).

2.5. Statistical analysis

The results of LC50 and EC50 were assessed by the PROBIT analysis (US-EPA software ver 1.5). On the other hand, the frequency of abnormal oocytes/embryos was evaluated and the embryotoxic and teratogenic effects, as well as the heart rate, were analyzed by the Fisher's exact test ($p < 0.05$) SPSS v10 (SPSS, Chicago IL). Egg batches were only used when the fertilization rate was above 90% and control groups showed less than 10% of teratogenicity at 96 hpf. Also, results of oxidative stress biomarkers were evaluated by one-way ANOVA to test differences among treatments (control, isolated forms and mixture) followed by a Tukey HSD multiple comparison test ($p < 0.05$) with the Program SPSS v10 (SPSS, Chicago IL). Correlations between biomarkers and the frequency of malformations were evaluated by using the Pearson's correlation test (SPSS, Chicago IL).

3. Results

3.1. Embryolethality data of isolated forms and the binary mixture of IBU and Al

Percentage values of mortality and malformations of embryos exposed to IBU, Al and the binary mixture are shown in Table 1. The number of malformations and deaths increased in a dose-dependent manner, except for the highest concentrations of IBU (11 and 20 μg L^{-1}) that showed a decrease in the total number of embryos with malformations. The LC50 values of IBU and Al were 8.06 μg L^{-1} (95% CI: 6.32–9.45) and 5.0 mg L^{-1} (95% CI: 3.99–6.47) respectively, while the EC50 values were 2.85 μg L^{-1} (95% CI: 1.47–3.54) and 3.58 mg L^{-1} (95% CI: 2.27–4.13). Teratogenic Index values were 2.82 for IBU and 1.39 for Al. Based on the TIs values that ranged between 1.39 and 2.82, both substances can be categorized as teratogenic with a relatively strong embryotoxic potential especially in the case of Al (Weigt et al., 2011). Embryos exposed to a mixture consisting of the EC50 values of both IBU and Al showed percentages of mortality and teratogenic effects higher than 50%; therefore, it is possible to infer that the combination of both substances is potentially toxic to *D. rerio* at the early stages of development.

Fig. 1 shows the live, dead, and total malformed embryos. The rate of live embryos decreased proportionally as concentrations of both substances increased; on the other hand, mortality and malformations increased as higher concentrations were reached. In the case of the exposure to the mixture, mortality and teratogenic values were higher than those obtained for live and normal embryos, and malformations were multiple, and more severe than isolated forms.

3.2. Main teratogenic effects and morphological alterations to embryonic development induce after exposure to isolated forms and mixtures of IBU and Al

Several malformations were observed after treatments with isolated forms and a mixture of IBU and Al (Fig. 2). The most frequent malformations induced by isolated IBU were delay in the hatching process, tail and head malformations, severe cases of scoliosis, lack of pectoral fin,

Table 1
Percentages of dead and total teratogenic embryos after exposure to IBU and Al isolated forms and mixtures.

Substance	Exposure concentration	Embryos exposed (20 oocytes per concentration, five replicates)	Mortality (%)	Embryos with teratogenic effects (%)
Ibuprofen ($\mu g\ L^{-1}$)	0	100	0	0
	0.1	100	11.0	38.0
	0.5	100	18.0	41.6
	1	100	26.0	46.0
	3	100	32.0	52.0
	5	100	38.0	55.0
	7	100	43.0	58.0
	9	100	58.0	65.0
	11	100	67.0	56.0
	20	100	76.0	48.0
			LC50 = 8.06 CI = [6.32–9.45] **TI = 2.82**	**EC50 = 2.85** CI = [1.47–3.54]
Aluminum ($mg\ L^{-1}$)	0	100	0	0
	0.01	100	16.0	30.0
	0.05	100	20.0	33.0
	0.1	100	25.0	36.0
	1	100	28.0	45.0
	2	100	36.0	48.0
	4	100	41.0	53.0
	6	100	53.0	70.0
	8	100	68.0	73.0
			LC50 = 5.0 CI = [3.99–6.47] **TI = 1.39**	**EC50 = 3.58** CI = [2.27–4.13]
Mixture		100	52.0	56.0

and pericardial edema. The main alterations induced by Al were hatching delay, hypopigmentation, tail and head malformation, lack of pectoral fin and buccal protuberance as well as pericardial edema. Hatching alterations, tail malformation, pericardial edema, head deformation, hypopigmentation, lack of pectoral fin, absence of buccal protuberance and developmental delay were the most common disorders detected for the mixture in which case, malformations were multiple and relatively more severe in comparison to the isolated forms, putting the life of embryos at risk.

Table 2 shows the photographs of the main malformations at different exposure times. As can be seen, the number and severity of malformations increased in time. At the highest concentrations, mortality increased due to the severity of malformations. Percentages of the most frequent malformations of the calculated additive interactions among IBU and Al and the actual values obtained after exposure to isolated forms and the mixture are listed in Table 3. The results show that isolated forms and the mixture induce additive values in all general endpoints and were significantly increased in comparison to the control group. Further, the mixture showed additive interaction, and were significantly higher concerning the isolated forms of both substances. Developmental delay, hypopigmentation and head malformation percentages obtained after exposure to the mixture were higher than individual substances and can infer an additive interaction. However, percentages of chorda and tail modified structure, pericardial edema and hatching alterations were significantly lower than additive interaction values.

3.3. Developmental score of D. rerio embryos after exposure to isolated forms and mixture of IBU and Al

D. rerio embryos exhibited manifold and specific deformities after exposure to isolated forms and the mixture. The number of embryos that showed morphological abnormalities (organisms with evident teratogenic effects) and the ones that presented any alteration of development when the score measure was applied, was dose-dependent at all times of exposure. Fig. 3 shows statistically significant ($p \leq 0.05$) changes between the concentration-response curves of embryos exposed to IBU, Al and the mixture in comparison to the control group, demonstrating that there is a continuous and gradual effect on the development of the embryos after exposure to environmentally relevant concentrations of both substances. Statistically significant decreases ($p \leq 0.05$) in the developmental score were observed at the highest concentrations of isolated forms and after exposure to the mixture, suggesting that these two substances in combination may delay the normal developmental process of embryogenesis.

3.4. Heart rate assessment

To determine the cardiac impairment caused by the exposure on zebrafish larvae, the heart rate determination was performed. After embryos were exposed to various concentrations of IBU and Al we found that heart rates in the larvae (72 hpf) decreased in a dose-dependent manner in comparison to the control group Fig. 4. The mixture also showed a statistically significant decrease when compared to the control group. These results support the hypothesis that IBU, Al and the mixture may act as cardiotoxins on early-staged zebrafish because of heart rate alterations, nevertheless, additional research is needed to fully identify the specific individual and interactive mechanisms involved in this endpoint.

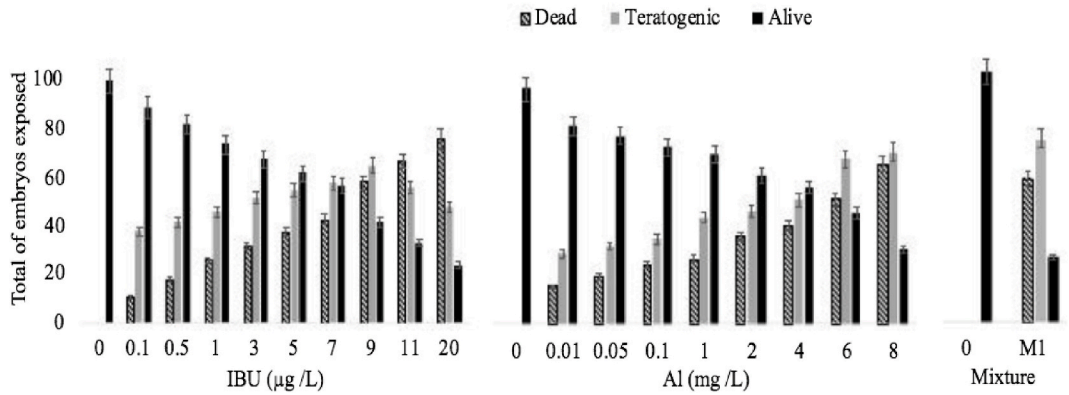


Fig. 1. Survival, mortality and malformations quantified on embryos exposed to IBU, Al and the mixture. Fisher's test ($p < 0.05$), $n = 100$ per concentration (20 oocytes per concentration, five replicates).

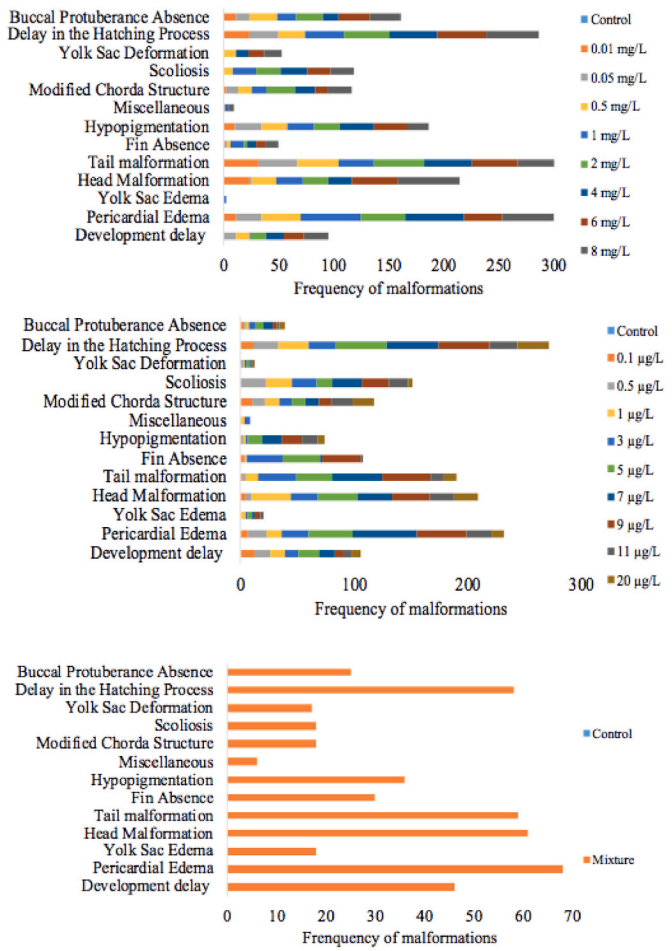


Fig. 2. Frequency of malformations induced by isolated forms of Ibuprofen(a), Aluminum (b) and the mixture (c) on *D. rerio* embryos. Fisher's Exact Test ($p < 0.05$), $n = 100$ per concentration (20 oocytes per concentration, five replicates).

3.5. Oxidative stress biomarkers

Fig. 5 and Fig. 6 show the results of oxidative stress biomarkers at 72 and 96 hpf. As observed, values of cell oxidation biomarkers including hydroperoxides, protein carbonyl content and lipid peroxidation increased in a time and dose-dependent manner. On the other hand, results of antioxidant activity showed a variable behavior regarding the control group ($p < 0.05$). In Table 5 (Supplementary data) correlations between frequency of major teratogenic effects and biomarkers of OS are shown. On that subject, OS biomarkers are strongly associated with the frequency of malformations observed after exposure to IBU and Al isolated forms and their mixture. These results support the hypothesis that oxidative stress is an important mechanism involved in the induction of toxic effects on *D. rerio* embryos.

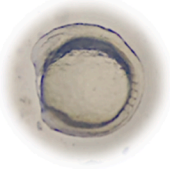
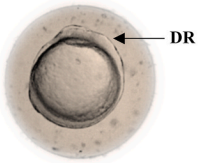
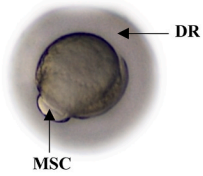
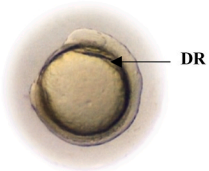
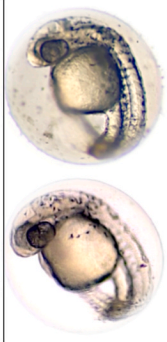
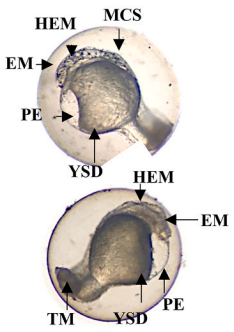
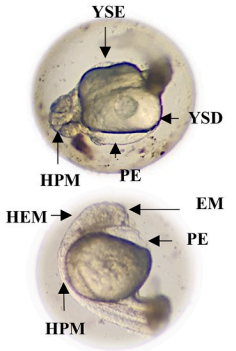
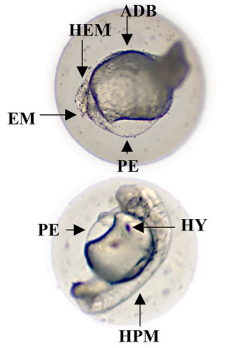
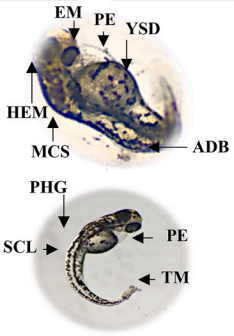
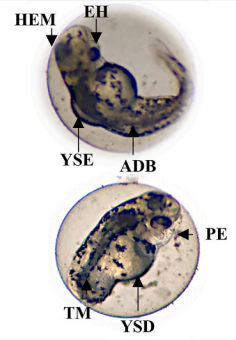
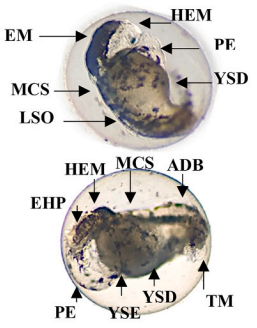
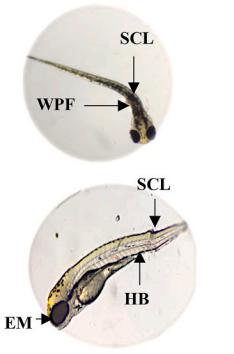
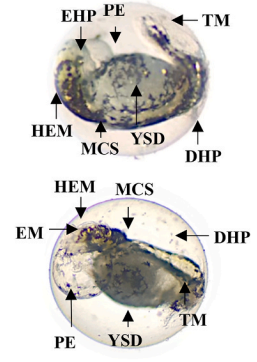
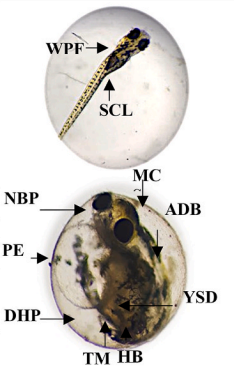
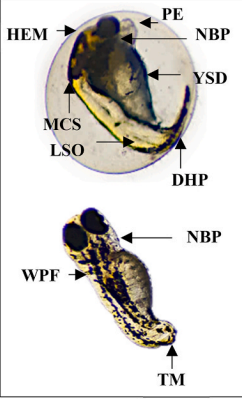
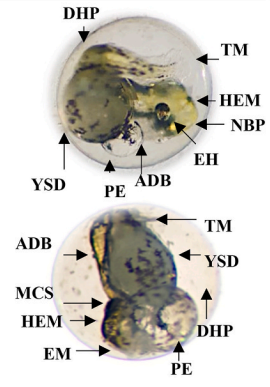
4. Discussion

Exposure to pharmaceutical compounds and metals promotes several toxicity mechanisms in aquatic species (Abdalla et al., 2019; Sivakumar et al., 2012). Many studies have been carried out on the different effects that these compounds can produce by themselves when they enter the environment and encounter non-target organisms; however, we will find a complex mix of all the different pollutants converging in an environment under natural conditions effluent. Therefore, the information on the effects, mechanisms of toxicity, and interactions in these mixtures are essential and currently scarce. Therefore, the present study aimed to evaluate whether exposure to isolated forms and the binary mixture of ibuprofen and aluminum-induced toxic effects on *D. rerio* oocytes; for which we opted to use different toxicity biomarkers that would give us a general overview of the damage that could occur in early stages of development, that is, embryotoxicity, teratogenesis, and heart rate; as well as oxidative damage and antioxidant activity, in order to be able to glimpse a mechanism of damage through oxidative stress.

In Mexico, regulatory authorities have established permitted maximum concentrations of Al for drinking water (0.02 mg L^{-1}) and aquatic life protection (0.05–0.1 mg L^{-1}) (SEDUE, 1989); nevertheless, as in many other countries, the presence of pharmaceuticals in aquatic ecosystems remains unregulated; among them, the occurrence of NSAIDs has become a matter of concern to the scientific community due to their harmful environmental effects since they adversely influence the aquatic and terrestrial biocenosis at various trophic levels (Islas-Flores et al., 2013; Jeffries et al., 2015; Luja-Mondragón et al., 2019). IBU is a propionic acid derivate (Montes et al., 2016) known to intervene in the synthesis of prostaglandins (PGE_2) (Manku et al., 2019; Wagner et al., 2019) and is considered the third most consumed non-steroidal anti-inflammatory drug worldwide (Brun et al., 2006). Due to the above, in our study, we used environmentally relevant concentrations to know the effect these pollutants could have in isolation and a mixture.

The results concerning the embryotoxicity of IBU show that the 96-h LC50, EC50, and TI were 8.06 µg L^{-1}, 2.85 µg L^{-1}, and 2.82, respectively. In previous studies, our research group reported similar results in another teleostean species; on common carp oocytes, Gutiérrez-Noya et al. (2020) found an LC50 of 4.17 µg L^{-1}, EC50 of 1.39 µg L^{-1}, and TI of 3.0 after exposure to environmentally relevant concentrations (1.5–11.5 µg L^{-1}). Further, Luja-Mondragón et al. (2019) reported an LC50 of 5.65%, EC50 of 3.85% µg L^{-1}, and TI of 1.46 at 96 hpf exposure to a hospital effluent containing NSAIDs among other pharmaceutical products. This substance can be categorized as potentially teratogenic based on the TI value calculated for IBU (2.82). These may be due to IBU undergoing biotic and abiotic reactions due to environmental conditions and diverse cytochrome P450 (CYP) enzymes (Gagné et al., 2006; Islas-Flores et al., 2017). According to Jones et al. (2021) *Danio rerio* larvae can metabolize IBU to a product considered a significant metabolite in mammals (hydroxy-ibuprofen parent), suggesting that the CYP2C9 and CYP2C19 isoforms are also present in zebrafish tissues at early stages of development; reinforcing with this what was said by Davies in 1998 when found that fish can metabolize IBP like mammals. This biotransformation results in the formation of smaller, more hydrophobic molecules that have been reported to have higher toxicity than IBU parent in a variety of organisms (Miranda et al., 1991); by ROS formation as a result of the redox cycling (Ahmad et al., 2000; Abdollahi et al., 2004). On the other hand, heavy metals induce alteration in the hatching process, developmental abnormalities during the organogenesis phase, and a decrease in the survival of the hatched larvae (Jezierska et al., 2009). The results of embryo lethality found in this study demonstrate that exposure to Al at environmentally relevant concentrations and concentrations considered safe for aquatic life protection (SEDUE, 1989) are proven to be toxic for oocytes of *D. rerio*; and the LC50, EC50, and TI of 5.0 mg L^{-1}, 3.58 mg L^{-1} and 1.39 respectively, show that this metal can be classified as a teratogenic compound with a strong embryo lethal character (Lee et al., 2013; Weigt et al., 2011). Al is considered non-essential for the biota; its bioavailability is strongly affected by physicochemical parameters of the aquatic environment (Santore et al., 2018; Trenfield et al., 2012). Moreover, even though it is known that due to its stable oxidation state, this metal has no redox activity in biological systems, is considered a strong pro-oxidant ion (Abreo et al., 2004; Fernández-Dávila et al., 2012) capable of disrupting ion-metal metabolism promoting the Fenton reaction and leading to the oxidation of main biomolecules (Ruipérez et al., 2012; Yousef, 2004) which may explain the observed results. Other studies report that adult teleost fish *Cirrhinus mrigala*, *Geophagus brasiliensis*, *O. mossambicus*, and *M. salmoides* presented high concentrations of Al in several tissues suggesting that this metal bioaccumulates after exposure (Oberholster et al.,

Table 2
Representative photographs of main malformations found in *Danio rerio* embryos after exposure to IBU, Al and the binary mixture.

hpf	Malformations			
	Control	IBU	Al	Mixture
12				
24				
48				
72				
96				

ADB = Abnormal development of the body; **DHP** = delay in the hatching process; **DR** = development retardation **EHP** = eye hypoplasia; **EM** = eye malformation; **HB**= Hemorrhaging in the body; **HEM**= Head malformation; **HPM** = hypopigmentation; **HY**= Hemorrhaging in the Yolk; **MCS** = modified chorda structure; **MSC** = Miscellaneous; **NBP** = no buccal protuberance; **PE** = pericardial edema; **PHG** = premature hatching; **SCL** = scoliosis; **SMD** = somite dysmorphology; **TM** = tail malformation; **WPF** = without pectoral fin; **YSE** = yolk sac edema; **YSD** = yolk sac deformation.

Table 3
Percentages of the most frequent malformations obtained after exposure to isolated forms and the mixture of IBU and Al and calculated additive interactions.

Malformations	Control	Ibuprofen	Aluminum	Additive interaction	Mixture
-Developmental delay	0%	14.5% [a,d,e]	16.25% [a,d,e]	30.75%	43.3% [a,b,c,d]
-Head malformation	0%	21.5% [a,d,e]	21.25% [a,d,e]	42.75%	60% [a,b,c,d]
-Chorda and tail modified structure	0%	36.9% [a,d,e]	40.9%[a,d,e]	77.8%	56% [a,b,c,d]
-Hypopigmentation	0%	7.3% [a,c,d,e]	21.87% [a,b,d,e]	29.17%	36% [a,b,c,d]
-Pericardial edema	0%	37% [a,d,e]	38.3% [a,d,e]	75.3%	66.6% [a,b,c,d]
-Hatching alterations	0%	36.8% [a,b,d,e]	40.83%[a,d,e]	77.63%	58.3% [a,b,c,d]

ANOVA and Tukey HSD Test. ($p < 0.05$).
[a] Significant difference regarding the control group.
[b] Significant difference regarding the Ibuprofen treated group.
[c] Significant difference regarding the Aluminum treated group.
[d] Significant difference regarding the Additive interaction.
[e] Significant difference regarding the Mixture treated group.

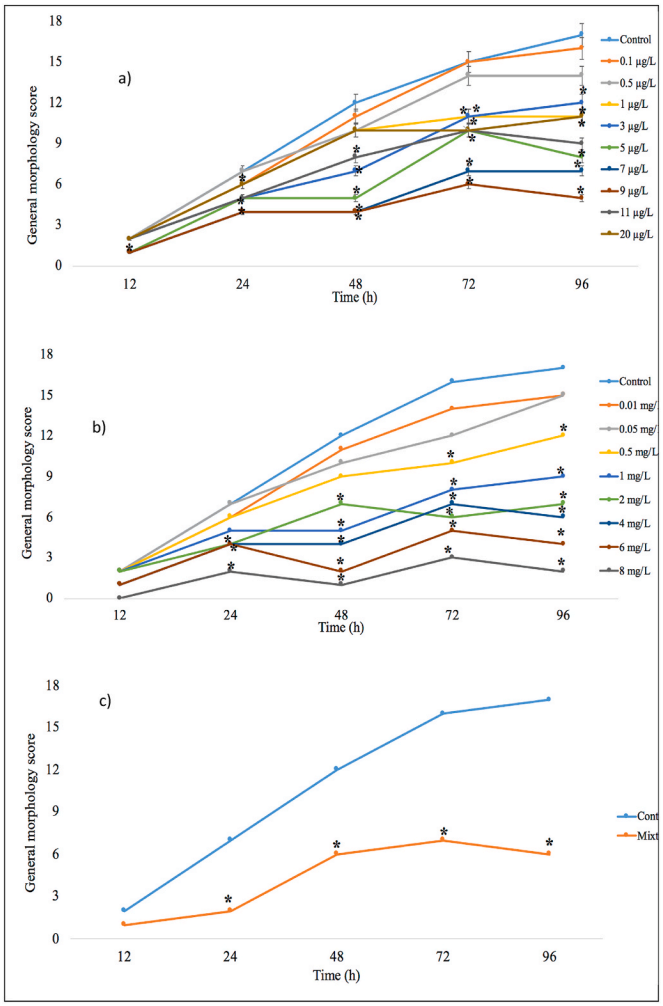


Fig. 3. Concentration-response curves of IBU(a), Al (b) and the mixture (c) on *D. rerio* embryos. * Significant difference regarding the control group. Fisher's Test ($p < 0.05$), $n = 100$ per concentration (20 oocytes per concentration, five replicates).

2012; Sivakumar et al., 2012; Voigt et al., 2015). Gopalakrishnan et al. (2008) reported the capability of oocytes and larvae to bioaccumulate heavy metals. It has been demonstrated that the chorion is unable to fully protect the embryo against metal ions penetration, mainly during the egg's swelling; thus, the accumulation of metal in oocytes is possible and could contribute to its toxic effect (Fent et al., 2010; Jezierska et al., 2009).

However, as TI value does not consider the concentration-response curves or the proportion of organisms with lethal and teratogenic effects, and therefore, it is an estimate of the teratogenic effects; meticulous observations and measurement of endpoints determined in this study were made in order to be able to assess the toxicity of the compounds (Weigt et al., 2011).

Our results showed that IBU also induced hatching alterations, skeletal malformations, and cardiac impairment, showing that this NSAID has the potential to induce significant malformations at environmentally relevant concentrations. Different mechanisms of embryotoxic action have been identified after exposure to this pharmaceutical compound. Proteratogen substances can be biologically activated via oxidation to toxic metabolites such as electrophilic molecules or free radical by-products due to a phenomenon termed oxidative stress (OS) (Wells et al., 2005). OS is considered an imbalance between pro-oxidant species and antioxidant defenses due to changes in the normal REDOX intracellular environment (Abdelkhalek and Ghazy, 2014; Ganesan et al., 2016; Hansen et al., 2018; Poprac et al., 2017; Rodríguez-Sánchez et al., 2012). This mechanism has been associated with a broad spectrum of birth defects, including skeletal aberrations, cardiovascular alterations, and delays in embryonic development (Kovacic Laher, 2014; Pašková et al., 2011). Reactive oxygen, nitrogen, and sulfur species have been largely recognized as influencing the transcriptional activation of genes involved in regulating cellular activities hence playing essential roles in the development of vertebrates (Covarrubias et al., 2008; Mugoni et al., 2014; Paulsen and Carroll, 2013). Changes in the redox system have been related to noxious effects during embryonic development and severe malformations in fish species, as observed (Wells et al., 2005). Moreover, biotransformation of IBU into electrophilic by-products like 2-OH-IBU, *p*-benzoquinones, and IBU-derived acyl glucuronides may elicit oxidative damage to biomolecules through the indirect formation of ROS via redox cycling in higher vertebrates (Barata et al., 2005; Boelsterli, 2003). Also, our results can be explained by the MOA of NSAIDs, involved in the inhibition of the cyclooxygenase (COX-1 and COX-2) system, a group of enzymes that catalyze a key step in the conversion of arachidonic acid into prostaglandins (PGH_2) (Brausch et al., 2012); because the embryogenesis is strongly affected by

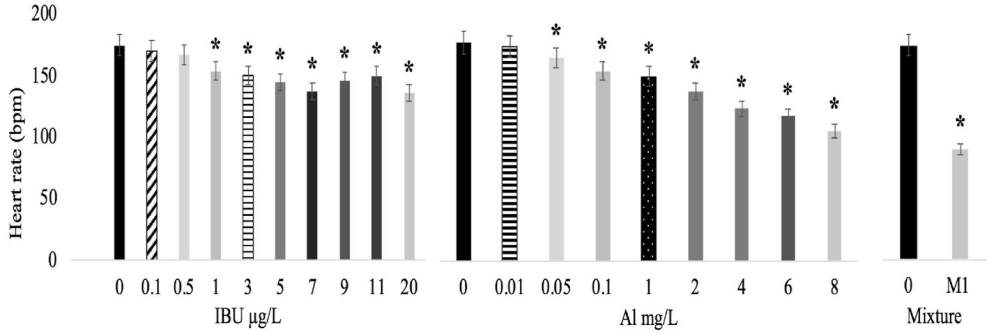


Fig. 4. The heart rate of zebrafish larvae (72 hpf) exposed to different concentrations of ibuprofen, aluminum, and the binary mixture. * Significant difference regarding the control group. Fisher's Test ($p < 0.05$), $n = 30$ per concentration.

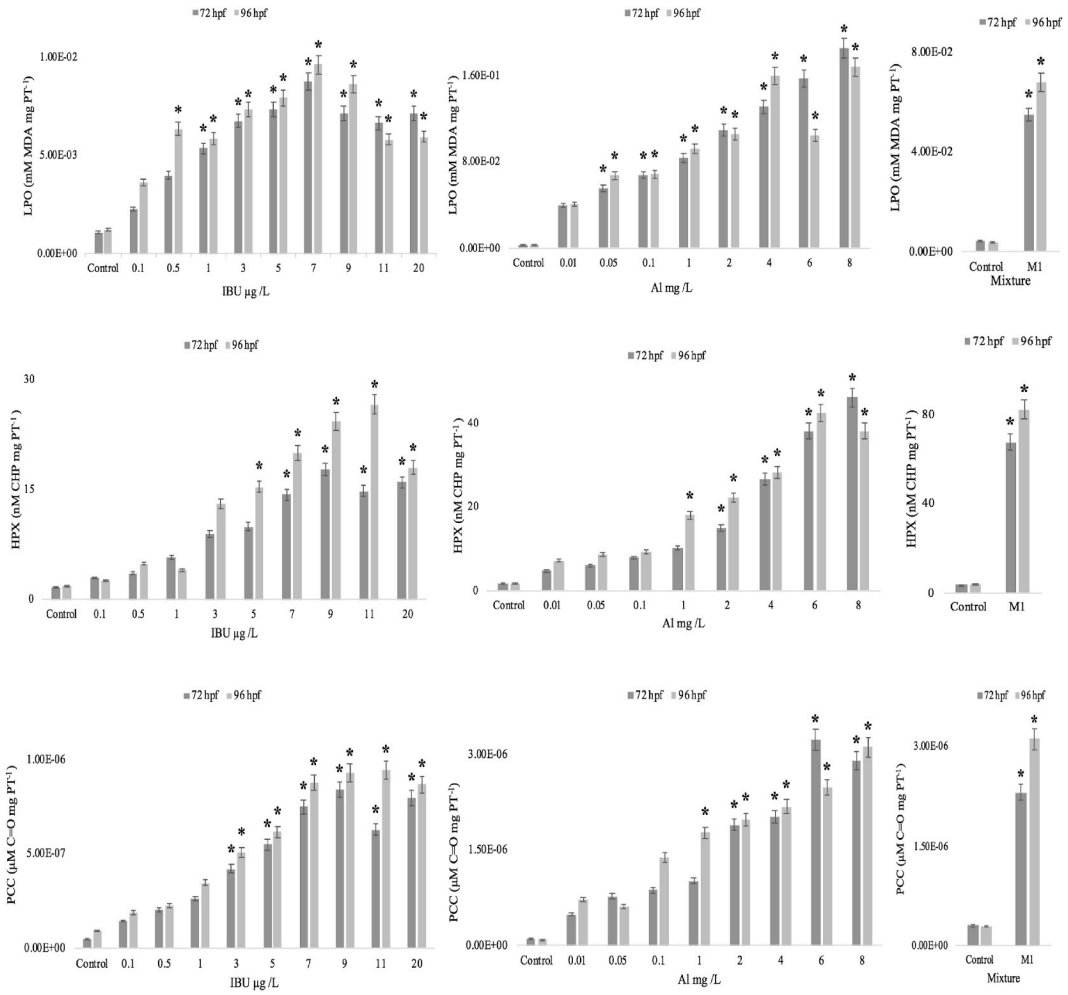


Fig. 5. Cellular oxidation biomarkers (a) Lipid Peroxidation (LPO), (b) Hydroperoxides Content (HPX) and (c) Protein Carbonyl Content (PCC) evaluated in *D. rerio* embryos at 72 and 96 hpf, exposed to isolated forms of ibuprofen, aluminum, and the binary mixture (M1). *Significant difference concerning the control group. ANOVA, Tukey HSD ($p < 0.05$).

the synthesis of prostaglandins and cyclooxygenase signaling (Cha et al., 2006). Prostanoids have been considered to participate in the reproductive cycle actively, cardiovascular homeostasis, blood vessel and kidney development, and chondro- and osteogenesis (Peltzer et al., 2019). The inhibition of the cyclooxygenase signaling after the gastrulation period results in the defective formation of the vascular tube, alterations in the intersomic vessels in the posterior region of the body, growth arrest, skeletal defects and malformation of the nephric duct in zebrafish embryos (Cha et al., 2005). In the present study, a delay in the hatching process at 72 hpf was observed. Before hatching, the chorion starts a softening process due to the proteolytic activity of the chorionase enzyme enhancing the permeability and thus increasing the adsorption of different substances from the surroundings (Tenorio-Chávez et al., 2020). Alterations in the hatching process can be due to the inhibited activity of the chorionase, osmotic disturbances affecting the proteolytic activity of the enzyme, elevated consumption of oxygen during the swelling phase and behavioral disturbances that result in depleted movements necessary to the mechanical rupture of the membrane with

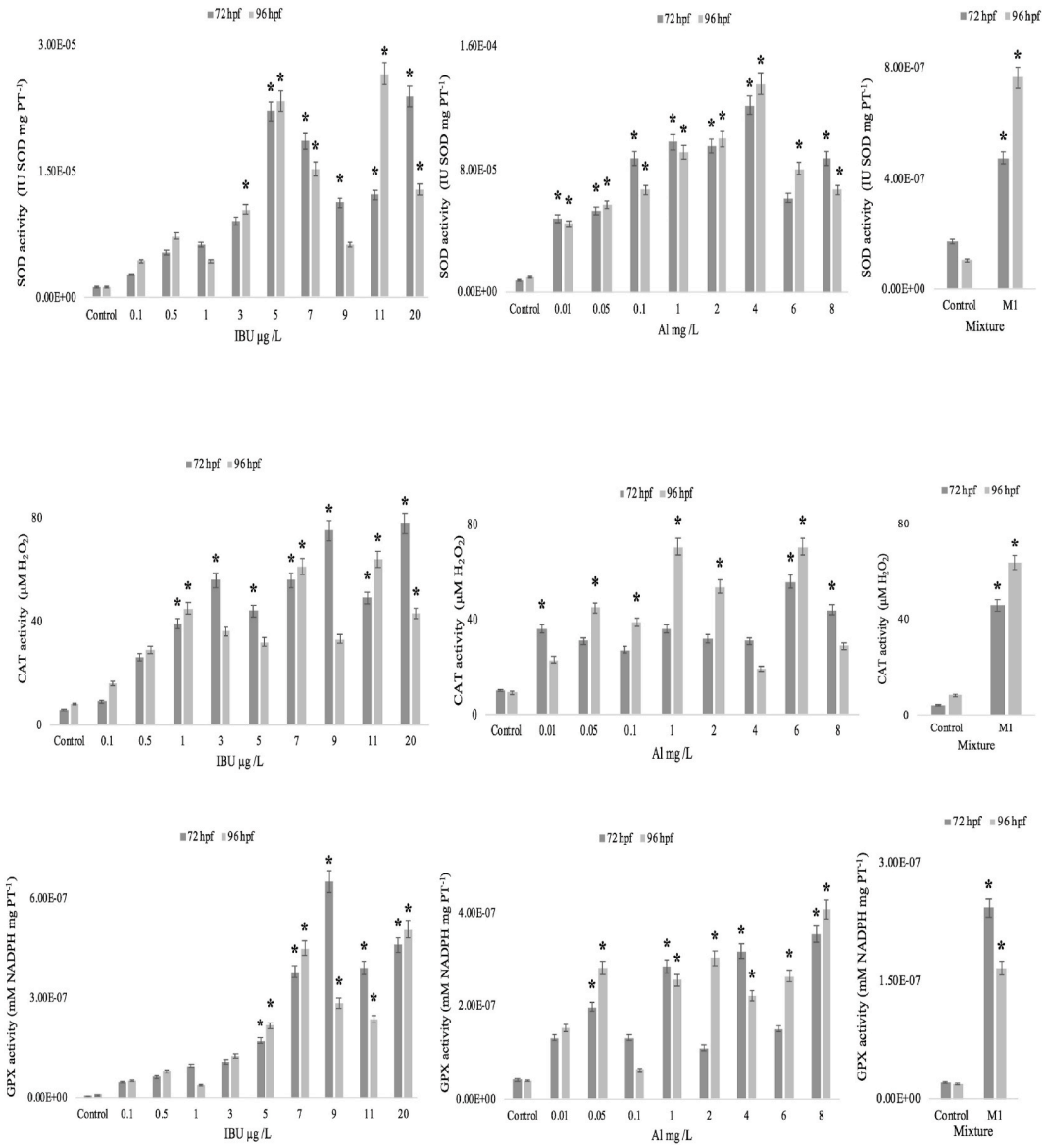


Fig. 6. Antioxidant activity biomarkers (a) Superoxide dismutase (SOD), (b) Catalase (CAT) and (c) Glutathione Peroxidase (GPX) evaluated in *D. rerio* embryos at 72 and 96 hpf, exposed to isolated forms of ibuprofen, aluminum, and the binary mixture (M1).*Significant difference concerning the control group.ANOVA, Tukey HSD ($p < 0.05$).

the tail (Haendel et al., 2004; Strmac, 2002).

In addition to this, Al is known to impair cellular functions by altering the structure of proteins through its high affinity to sulfhydryl groups found in the proteins possessing sulfur amino groups like cysteine (Khan, 2013). Once this metal penetrates the chorion, it can bind the thiol groups of proteins and induce the formation of radical species, responsible for embryotoxic effects in various aquatic organisms (Adeyemi et al., 2015; Ganesan et al., 2016). García-Medina et al. (2010) and Hasan et al. (2018) reported that this metal affects electron transport in the respiratory chain because of direct damage to mitochondria and interact in a direct (ion-protein) and indirect (protein-protein) way with various proteins and enzymes essential for several biological processes as well as molecular functions in different organisms. Further, skeletal abnormalities, mainly tail and craniofacial malformations found in the present study may be explained as Al may accumulate in bones as it displaces calcium on the bone surface, leading to aberrations in mineralization, hypercalcemia, hypercalciuria, and osteomalacia. Those effects can affect heart activity, the nervous system, and osteogenesis in embryos via inhibition of the Wnt/β-catenin signaling pathway, promoting axial malformations like scoliosis, kyphosis, and lordosis (Brannen et al., 2010; Klein, 2019; Song et al., 2017; Sun et al., 2017). It can be speculated that the reduction in the hatching rate observed in the present study results from the interaction of aluminum with the chorionase enzyme, a metalloprotease named hatching protease (Scheil et al., 2009). Hypopigmentation was also observed on embryos exposed to Al. It has been reported that metals generate a decrease in the expression of key genes involved in the formation of the neural crest, a structure from which peripheral neurons, glia, elements of the craniofacial skeleton, and pigment cells are derived (Rocha et al., 2020). The adequate development and maintenance of the neural crest have been associated with retinoic acid signaling (Chawla et al., 2018). Retinoic acid (RA) is a mighty factor during embryogenesis, mainly during gastrulation and later organogenesis, since it participates in the growth, differentiation, and morphology of various cells (Kawakami et al., 2005). Alterations in its signaling caused by Al are associated with malformations of the skeletal, ocular, CNS, and cardiovascular systems (Szutowicz et al., 2015). Our study is consistent with Tle et al. (2012), who found that early RA deprivation resulted in a deficiency of

pigmented cells in eyes of zebrafish embryos.

As for the heart rate impairment observed, in the case of the highest concentrations of IBU, it may be due to the inhibition of the expression of COX-1 and therefore the prostaglandin signaling pathway during the development of vasculature, arterio-venous differentiation, and angiogenesis process that control the cardiovascular homeostasis (Cha et al., 2005). Also, heart rate alteration can be considered a secondary effect of pericardial edema formation due to the mechanical compression affecting blood circulation and presumably generating alterations in heart morphology (Antkiewicz et al., 2005). In general, our results are consistent with other authors who referred that IBU generates severe cases of scoliosis, notochord and tail malformations, pericardial edema, developmental delay, pectoral fin defect, decrease in the heart rate, and behavioral disturbances on *D. rerio* and *C. carpio* embryos at concentrations between 1.5 and 100 μg L^{-1} (David and Pancharatna, 2009; Gutiérrez-Noya et al., 2020). Besides, it has been demonstrated that some pharmaceuticals that act as inhibitors of the eicosanoids biosynthesis can impair the cellular and humoral immune response as well as the maintenance of the oxidative homeostasis in different organisms (Büyükgüzel et al., 2010, 2007). On the other hand, in the case of Al, a different effect was observed; heartbeat frequency was decreased in embryos exposed after 72 hpf. Two major targets have been identified for the toxic action of Al, the nervous and circulatory systems (Monaco et al., 2017). Several mechanisms of cardiovascular alterations caused by aluminum have been identified, including direct toxicity on cardiac myocytes, inhibition of the cytochrome C oxidase, excessive loss of fluids, adrenal gland toxicity, oxidative stress, impairment in mitochondrial activity, and apoptosis processes, among others (Asghari et al., 2017; Mohan et al., 2015). Following this study, Monaco et al. (2017) reported pericardial edema and impaired heart rate in zebrafish embryos after exposure to 100 mM of Al.

Thus, all of this modes of action of Al and IBP in isolated form, can explain the results of embryos exposed to the mixture, that showed percentages of mortality and teratogenic effects higher than therefore, and make it possible to infer that the combination of both substances is additive in *D. rerio* at the early stages of development. There are three common models of mixture toxicity: concentration addition (combined effect will be the sum the effects by each compound alone), subtraction (for compounds with different mechanisms of action the combined effects may be less), and synergy (the combined effects is more than the sum of the effects by each compound alone) (Cleuvers, 2003; Cleuvers and Ratte, 2002).

As mentioned before, environmental pollution occurs in the form of a mixture and interactions may occur, so any variation in the biomarkers evaluated can be due to alterations of their mechanisms of toxicity (Luja-Mondragón et al., 2019; Pérez-Alvarez et al., 2018). Thus, results obtained after exposure to the mixture consisting of the EC50 values of both substances show that the number of dead and malformed organisms was higher than 50% and 70%, respectively, of the total embryos, evaluated. Besides, we detected that the mixture induced severe dysmorphology of various structures. The most frequent malformations detected after exposure to the mixture were hatching delays, axial defects including the tail, head, and chorda alterations, pericardial edema, hypopigmentation, and developmental delay, as well as cardiac impairment. Synergistic effects can be considered when the effect of the exposure to a mixture seems to be higher than or distinct than the one expected by the additive response; on the other hand, antagonism appears when one or several compounds that take part in a mixture interfere with the action of other, thus there is a decrease in the predicted effect of each compound with similar or different structures (López González et al., 2019; Quiroga-Santos et al., 2021). Additive interaction values of frequency of malformations (Table 3) show that developmental delay, hypopigmentation, and head malformation exhibited synergic effects concerning the actual values obtained after exposure to the mixture. On the other hand, endpoints such as chorda and tail defects, hatching delay, and pericardial edema led to significantly lower results as indicatives of an antagonistic effect of both substances. Nevertheless, the qualitative observation of the embryos and larvae showed that even though the frequency of malformations fluctuated in comparison to the isolated forms, the developmental alterations induced by the mixture seem to be more severe in all evaluated endpoints putting the life of the organisms at risk. Following the results of our experiment, other studies evaluating hospital effluents containing mixtures of pharmaceuticals and metals detected severe malformations, including microcephaly, damage to the notochord, tail, fin, and gut, as well as hatching alterations, pericardial edema, axial malformations, and delay in the development have been reported in *X. laevis* and *D. rerio* respectively (Pérez-Alvarez et al., 2018; Tenorio-Chávez et al., 2020).

5. Conclusions

General toxicity pathways of many environmental pollutants are mainly mediated through interactions as no substances are in isolation in the environment; therefore, the study of complex mixtures should be considered relevant. This study highlighted the importance of researching the potential environmental effects that might arise when active pharmaceutical ingredients and micropollutants from other sources, such as metals, converge and interact in aquatic systems. In conclusion, ibuprofen, and aluminum at environmentally relevant concentrations, act as embryotoxic and teratogenic agents on *D. rerio*. The toxic response of the mixture was different in number and severity compared to the isolated forms, and an additive type interaction was observed. The results showed that the different mechanisms of toxicity of IBU and Al, including oxidative stress, led to various patterns of malformations; thus, in addition to the embryotoxic potency determination and the identification of morphological and biochemical abnormalities, are a helpful tool to elucidate mechanisms of embryotoxicity further.

Author statement

Livier Sánchez-Aceves and Itzayana Pérez-Alvarez performed all the exposure experiments. Leobardo Manuel Gómez-Oliván and Livier Sánchez-Aceves were involved in the conception. Leobardo Manuel Gómez-Oliván and Livier Sánches-Aceves and Hariz Islas Flores were involved in the design and interpretation of the data and the writing of the manuscript with input from Damià Barcelò.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.118078.

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