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# Geno-cytotoxicity and congenital malformations produced by relevant environmental concentrations of aluminum, diclofenac and their mixture on *Cyprinus carpio*. An interactions study

Eldher Hissadam Quiroga-Santos [a], Marcela Galar-Martínez [a,*], Sandra García-Medina [a,*], Eloy Gasca-Pérez [b], Selene Cano-Viveros [a], Karina Ruíz-Lara [a], Leobardo Manuel Gómez-Oliván [c], Hariz Islas-Flores [c]

[a] *Laboratorio de Toxicología Acuática, Sección de Graduados e Investigación, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional. Unidad Profesional Adolfo López Mateos, Av. Wilfrido Massieu s/n y Cerrada de Manuel Stampa, Col. Industrial Vallejo, C.P. 007700, México D.F., Mexico*

[b] *Cátedra CONACYT, Laboratorio de Toxicología Acuática, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Unidad Profesional Adolfo López Mateos, México City, Mexico*

[c] *Universidad Autónoma del Estado de México, Facultad de Química, Departamento de Farmacia. Paseo Tollocan, esq. Paseo Colón, Toluca, Estado de México, C. P. 50100, Mexico*

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ABSTRACT

Several studies highlight the presence of aluminum and diclofenac in water bodies around the world and their ability to induce oxidative stress and a negative effect on biomolecules in several aquatic species. However, studies evaluating the toxic effect of mixtures of these contaminants are scarce. The objective of this work was to determine the genotoxic, cytotoxic and embryotoxic effect of the mixture of aluminum and diclofenac at environmentally relevant concentrations on *Cyprinus carpio*.

Juveniles of *Cyprinus carpio* were exposed to 0.31 μg $L^{-1}$ of diclofenac, 24.45 mg $L^{-1}$ of aluminum, and a mixture of both contaminants at the same concentrations for 12, 24, 48, 72 and 96 h. After the exposure time the liver, gills and blood were extracted and the following biomarkers were evaluated: micronucleus frequency, comet assay, caspase activity and TUNEL test. On the other hand, *Cyprinus carpio* embryos were exposed to diclofenac (0.31 μg $L^{-1}$), aluminum (0.06 mg $L^{-1}$) and their mixture at the same concentrations and exposure time. Microscopic observation was performed to evaluate embryonic development at 12, 24, 48, 72 and 96 h.

Diclofenac (0.31 μg $L^{-1}$) induces significant increases in micronucleus frequency with respect to control ($p < 0.05$), in all tissues. Aluminum (24.45 mg $L^{-1}$) significantly increases DNA damage index in liver and blood cells with respect to control ($p < 0.05$). All treatments increase caspases activity in all tissues with respect to control ($p < 0.05$). Diclofenac increases the percentage of TUNEL-positive cells in liver and blood; while aluminum and the mixture increases it significantly in gills and blood with respect to the control ($p < 0.05$). The mixture significantly delays embryonic development, while aluminum and the mixture significantly increase teratogenic index with respect to control ($p < 0.05$). In conclusion, exposure to environmental concentrations of aluminium, diclofenac and their mixture induces genotoxic damage, cell death by apoptosis and negative effects on the development of *Cyprinus carpio* and the toxic response is modified by the interaction of the xenobiotics.

## 1. Introduction

Anthropogenic activities are a constant threat to the stability of ecosystems, and in this sense, aquatic systems are receptors for all kinds of urban waste, including the presence of persistent toxic compounds in the water column and sediments (Rotter et al., 2015; Reichwaldt and Ghadouani, 2016).

Pharmaceuticals and metallic elements are groups of toxics widely distributed in water bodies. These agents and their metabolites can reach water bodies through domestic, hospital and industrial effluents,

* Corresponding authors at: Marcela Galar-Martínez, Laboratorio de Toxicología Acuática, Departamento de Farmacia, Escuela Nacional de Ciencias Biológicas, IPN, Unidad Profesional Adolfo López Mateos, Av. Wilfrido Massieu s/n y cerrada Manuel Stampa, Col. Industrial Vallejo, CP 07700, México D.F., Mexico.
*E-mail addresses:* mgalarm@ipn.mx (M. Galar-Martínez), sagarciam@ipn.mx (S. García-Medina).

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as well as agricultural activities and as a consequence of precipitations and acid drainage (Lydersen and Löfgren, 2002; Vystavna et al., 2013; Luja-Mondragón et al., 2019; Sanjuan-Reyes et al., 2020).

Diclofenac (DFC) is a non-steroidal anti-inflammatory, widely used for the treatment of joint pain and inflammation (Atzeni et al., 2018). It is a drug capable of causing acute toxicity and chronic effects on aquatic species, in addition to being inserted into trophic chains through bioaccumulation (Swan et al., 2006). DFC induces oxidative stress on aquatic species, as reported by Saucedo-Vence et al. (2014), who found that subacute exposure of *Cyprinus carpio* to a concentration of 7.098 mg $L^{-1}$ elevates the activity of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) and increases lipid peroxidation (LPx) levels. Gómez-Olivan et al. (2014) demonstrated that the subacute exposure of *Daphnia magna* to a concentration of 9.7 mg $L^{-1}$ of this drug also increases the antioxidant enzymatic activity of SOD and CAT, as well as the levels of LPx; it was also demonstrated that at these concentrations a genotoxic activity is induced after 48 h. On the other hand, a study on *Danio rerio* embryos conducted in order to determine the toxicity of DFC in their development, showed that concentrations of up to 100 mg $L^{-1}$ can cause embryos mortality rates rise up to 100 % (Hallare et al., 2004).

Aluminum (Al) is one of the main components of the Earth's crust of which it forms part in a proportion of 8%, and only surpassed among metals by silicon (26.5 %) (Exley, 2003). This is one of the most widely distributed metals in aquatic ecosystems, and its toxicity is variable due to the complexity of the physicochemical forms that are presented in solution. Although most chemical forms are not considered toxic, highly toxic inorganic monomeric species tend to form under special acid pH conditions. Such acidity condition is very common in water bodies due to excessive waste (García-Medina et al., 2011).

In fish, Al at concentrations higher than 10−15 μg $L^{-1}$ produces gill accumulation, increased secretion of the gill mucosa, respiratory problems, imbalance in the regulation of ions and decreased growth, also affects cell functions by interfering in cell differentiation, cell tissue integrity and increased production of free radicals that react with membrane lipids and generate changes in the transport of proteins (García-Medina et al., 2011; Poléo et al., 2017; Ramírez-Duarte et al., 2017).

Stephens and Ingram (2006), studied the mortality of *Bidyanus bidyanus*, *Carassius auratus* and *Cyprinus carpio* exposed to water with high concentrations of Al, and observed histological damage in the gills, consisted of diffuse hypertrophy, hyperplasia of the gill epithelium and fusion of secondary lamellae. In the liver, the damage was more severe, presenting diffuse fat degeneration of hepatocytes, as well as growth of the hepatocyte nuclei with marginalized chromatin.

García-Medina et al. (2011) found that in common carp, aluminum is capable of producing reactive oxygen species, and a reduction-oxidation (REDOX) imbalance that could induce damage to biomolecules. This could also affect cell integrity and induce malformations in carp embryos.

Even though reports about the individual toxic effect of DFC and Al have been written, studies on the toxic effects that both xenobiotics combined can cause are inexistent. This is important to consider, since most pollutants in the environment act as mixtures and not individually. This can cause toxicological interactions that modify the expected toxic response that these xenobiotics have while isolated (Hernández et al., 2017).

*Cyprinus carpio* is a fish widely used in aquaculture. It has been used as test organism in toxicological trials due to its economic importance and wide geographical distribution (Oruç and Uner, 2002).

The objective of this study was to determine the genotoxicity, cytotoxicity and embryotoxicity induced by the mixture of diclofenac and aluminum at environmentally relevant concentrations on *C. carpio*.

## 2. Materials and methods

### 2.1. Obtainment and acclimatization of C. carpio

Carps (*Cyprinus carpio*) in juvenile stage of at least 3 months of age, with a length of 19.05 ± 0.51 cm and a weight of 200 ± 2 g were obtained from the Tiacaque Aquaculture Center (Estado de México), and transported to the laboratory in plastic bags with properly oxygenated water. At the laboratory, fishes were distributed in glass aquariums equipped with filtration and aeration systems, and were kept at room temperature, with 8:16 h dark-light cycles and fed daily with high quality Nutripec® food. Water was kept between 20 ± 2° C, with oxygen saturation between 80–90 % and pH 7.5−8.0.

Embryos used in the embryotoxicity experiment were obtained by natural breeding and induced spawning at the Tiacaque Aquaculture Center (Estado de México). Fertilized embryos that did not exceed 4 hpf (hours post fertilization) were selected for the experiments.

### 2.2. Cyto-genotoxicity determination

Each trial was conformed of 6 fish groups, divided by exposure time (12, 24, 48, 72 and 96 h). This means a total of 30 fishes per trial were used. There were four exposure groups: two fish groups were placed in independent containers with an aluminum concentration of 24.45 mg $L^{-1}$ and a diclofenac concentration of 0.31 μg $L^{-1}$ respectively, in accordance with what was reported by González-González et al. (2014), who found these concentrations in the Madin dam, a freshwater body in the State of Mexico, Mexico. A third group was placed in a container with a mixture of both substances at those same concentrations. The fourth group was the control group, to which none of the toxic compounds studied were added.

After the exposure times, a blood sample was obtained by puncture of the caudal vein. Subsequently, the organisms were sacrificed in an ice bath with 2% lidocaine and the organs designated for the study (gills and liver) were dissected and processed to perform the tests for the evaluation of genotoxicity by determining the frequency of micronuclei (MN) and the test of unicellular electrophoresis (comet assay), and of cytotoxicity by means of the TUNEL test and the evaluation of the caspase-3 activity.

#### 2.2.1. Unicellular electrophoresis test (comet)

The evaluation of the genotoxicity of aluminum, diclofenac and mixture was performed using a comet test (Singh et al., 1988). This test evaluates the fragmentation of single strands of DNA (Costa et al., 2008). Liver, gills and blood cells were prepared through homogenization and filtering of tissues. The filtered cells were set on slides for the assay.

A 100 μL layer of 0.75 % low melting point agarose mixed with 50 μL of the cell homogenate was added to frosted slides and cooled in an ice bath. These preparations were immersed in a pH 10 lysis solution (2.5 M NaOH, 10 M EDTA, 10 M Tris with Triton and DMSO) for 1 h.

Subsequently, the preparations were placed in a horizontal electrophoresis chamber, containing a buffer solution (300 mM NaOH and 1 mM EDTA) at pH 13 and 4 °C, where they remained for 20 min. The electrophoresis was performed with a current of 25 V and 300 mA for 20 min. At the end of the electrophoresis, the preparations were removed from the chamber and neutralized with a tris regulator, pH 7.5 for 5 min.

Preparations were dyed with 50 μL of ethidium bromide (2 μg $mL^{-1}$). Sample readings were performed in a Motic ® BA410 epifluorescence microscope, equipped with a Motic ® XBE fluorescence lamp with a filter for wavelengths 510−560 nm, and the Motic Images Plus ® 2.0 image software. DNA comet tail length (T) and nucleoid diameter (N) were determined in order to establish the T/N index in units of 100 cells per concentration and time, for each experimental group.

#### 2.2.2. Micronuclei determination

Another of the biomarkers used to evaluate genotoxicity was the

frequency of MN. This test detects damage due to the breakage or delay of chromosomes. To determine the MN, smears of the cellular suspensions to analyze were performed (obtained with previous homogenization and filtration). The smears were fixed in pure ethanol for 3 min, left to dry and dyed with a Giemsa solution at 10 %. Finally, samples were washed with tap water. 2000 cells per treatment were quantized with a X52−146 binocular biologic microscope. Frequency was expressed as number of nucleated cells per 2000 cells (Cavas and Ergene-Gözükara, 2005).

The applied criteria for cell selection with micronuclei were: that small nuclei should not be linked to the main nuclei; color and intensity of the stain had to be similar to that of the main nuclei (Yong and Chang-Kee, 2006) and they should have had a diameter ratio between 1/5 and 1/20 of that of the main nuclei (Bolognesi et al., 2006).

#### 2.2.3. TUNEL-positive cells

The Millipore® ApopTag® Fluorescein S7110 kit was used to determine the percentage of TUNEL-positive cells. 300 µL of suspended cells in conservative solution were centrifuged at 3000 rpm for 5 min at 4 °C. The button was re-suspended in 50 µL of montage solution (PBM – liquid cytology kit). Later, 1 µL of cell suspension was placed on a polylisin slide and the preparation was allowed to dry at 60 °C for 5 min. Once dry, samples were immersed in cold acetone for 10 min, followed by a dehydration train of absolute alcohol at 96, 80, 70, 60 and 50 %, and water for 30 s each. Once cells were dehydrated, a proteinase K pretreatment (20 µg $mL^{-1}$) was performed for 10 min, the samples were washed three times with PBS for 10 min, and excess liquid discarded. 60 µL of equilibrium buffer from the ApopTag Fluorescein kit were added, and the samples were left to rest for 10 min. The TdT enzyme solution was added to the cells (65 µL per sample) and incubated in a humid chamber for 1 h at 37 °C. Reaction was interrupted with a wash/stop buffer for 10 min at room temperature. Samples were washed three times with PBS for 1 min. Then, anti-digoxin conjugate was added (65 µL per sample) in order to mark apoptotic cells by incubation in a humid chamber for 30 min at room temperature, protected from light. Sample was washed with PBS for 2 min at room temperature. Marking was contrasted with 1.5 µg $mL^{-1}$ of propidium iodure. Slides were observed in a Motic ® BA410 epifluorescence microscope, equipped with a Motic ® XBE fluorescence lamp with a filter for wavelengths 450−490 nm, and the Motic Images Plus ® 2.0 image software. Apoptosis was expressed as the number of TUNEL positive cells per 100 cells.

#### 2.2.4. Caspases activity

To determine the activity of caspase-3, a colorimetric test kit (CaspACETM Promega) was used, whose substrate (Acetyl-Asp-Glu-Val-Asp p-nitroaniline) binds to the enzyme releasing the chromophore p-nitroaniline (pNA). To quantify enzyme activity, pNA was determined in a spectrophotometer at 405 nm. The activity of caspase is expressed in µM of pNA released per hour per mg of protein compared to the control group. A reagent blank was prepared with 32 µL of caspase buffer (312.5 mM HEPES, pH 7.5; 31.25 % sucrose; 0.3125 % CHAPS (3-[(3-cholamido-propyl) dimethylammonio]-1-propane-sulfonate), 2 µL DMSO (dimethyl sulfoxide), 10 µL dithiothreitol (DTT; 100 mM) and 54 µL deionized water. For control and test groups, 32 µL of caspase buffer, 2 µL, 10 µL of DTT (100 mM), 20 µL of cell extract and 34 µL of deionized water were used. For positive control (hepatocytes, gill cells and tested blood with 1 µg de $Cd^{+2}$ per mL SFB of Invitrogene$^{MR}$) 32 µL of caspase buffer, 2 µL of DMSO, 10 µL of DTT (100 mM), 20 µL of cell extract and 34 µL of deionized water were used. For inhibited apoptosis (hepatocytes, gill cells and tested blood with 1 µg de $Cd^{+2}$ per mL SFB of Invitrogene$^{MR}$ and with 20 mM of Z-VAD-FMK by Promega), 32 µL of caspase buffer, 2 µL of DMSO, 10 µL of DTT (100 mM), 20 µL of cell extract with inhibited apoptosis and 34 µL of deionized water were used.

### 2.3. Embryotoxicity determination

Groups of 12 embryos, including a control group, were formed by triplicate. Test groups were exposed to 1000 µL of aluminum, diclofenac or the mixture at a concentration of 0.06 mg $L^{-1}$ and 0.31 µg $L^{-1}$ respectively, directly on 12 well plates and incubated for 96 h. Plates were kept at 28 °C ± 1 °C with natural light-dark cycles. Mortality rates in the control group were kept at a maximum of 10 % Test solutions, including control, were prepared with egg water as established by Westerfield (2007). The concentration of Al used in this part of the study was lower than that used in the study with juveniles (geno-cytotoxicity), because the latter caused the death of 100 % of the embryos, so for this experiment the organisms were exposed to one hundredth of the lowest concentration of aluminum found in the Madin Dam (0.06 mg $L^{-1}$) by González-González et al. (2014).

The morphological evaluation of the embryos was performed using an inverted microscope at 12, 24, 48, 72 and 96 h of exposure time. Each observation was recorded using a Microsoft® web cam, and the CyberLink YouCam® software. As described by Kimmel et al. (1995), an embryonic development scoring system was used and adapted for *Cyprinus carpio*.

The morphological characteristics to which the score was assigned were: tail development, somite formation, ocular formation, movement, blood circulation, heart beating, body-head pigmentation, tail pigmentation, pectoral fin appearance, mouth protuberance and hatching.

The teratogenesis index was calculated as established by Hermsen et al. (2011), determining 11 different types of malformations including: pericardial, ocular and vitelline sack edemas, head, otolith, tail, cord structure and heart malformations and vitelline sack scoliosis, raquisquisis and deformity. Teratogenesis index increases as the previously mentioned malformations do.

### 2.4. Statistical analysis

Comet, micronuclei, TUNEL and caspase activity results were analyzed using a Kruskal-Wallis non parametric test, followed by a multiple comparison using the Dunn method. Embryo development results were analyzed using a Friedman repeated measures analysis of variance on ranks with a Dunn multiple comparison. Regarding teratogenesis index, U-Mann Whitney non parametric test were used. All analyses were performed using $p < 0.05$ in the SigmaPlot 12® software.

## 3. Results

Fig. 1a shows the results concerning the comet test, where is observed that both Al and diclofenac, isolated or in mixture, produce the fragmentation of the single strands of DNA in the liver cells. These were significant ($p < 0.05$) for the group exposed to aluminum at 24 (23.4 %) and 48 h (25.26 %), diclofenac at 72 h (20.16 %) and the mixture at 24 and 96 h (19.34 % and 19.88 %). On gill tissue (Fig. 1b) none of the tested toxics, isolated or in mixture, produced changes in this parameter. Regarding blood tissue (Fig. 1c), a tendency to increase was observed at 24 h of exposure for all treatments with respect to control. This increase was particularly important at 24 (18.37 %) and 48 (23.98 %) hours of aluminum exposure, and in the group exposed to the mixture at 24 (20.27 %) and 72 h (22.40 %).

Damage by chromosomal break or delay in hepatic cells produced by the different treatments is presented in Fig. 2a. The increment of MN frequency was significant ($p < 0.05$) only for the group exposed to diclofenac after 48 and 72 h (946.69 and 1563.09 %), and aluminum after 48 h of exposure (809.5 %). The group exposed to the mixture showed a similar behavior with respect to control during the whole experiment. Gill tissue (Fig. 2b) showed a micronuclei frequency tendency to increase for the group exposed to aluminum, although this increase was not significant. On the other hand, the group exposed to diclofenac showed important increases during all exposure times

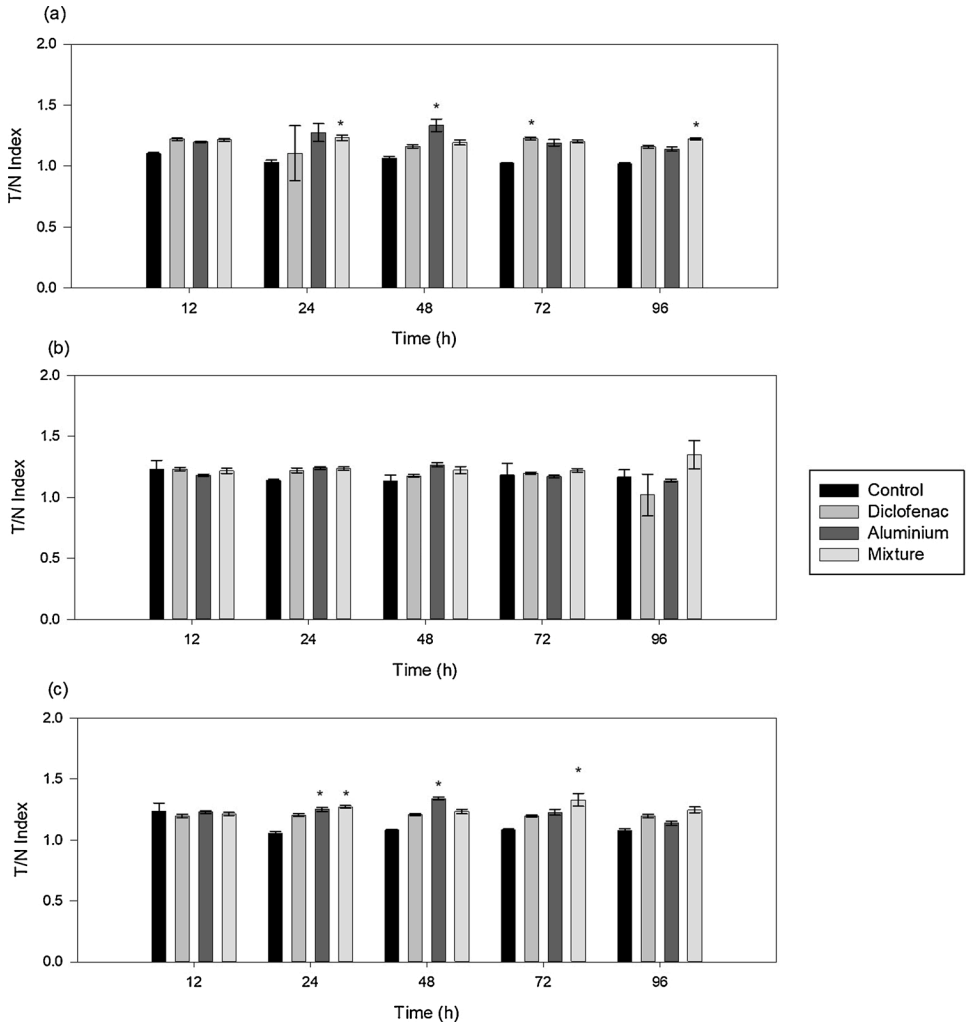


**Fig. 1.** DNA damage index in hepatic (a), gill (b) and blood (c) cells of *Cyprinus carpio* exposed to relevant environmental concentrations of diclofenac, aluminum and their mixture. * Significant statistical difference with respect to the control group. Statistical test: Kruscal- Wallis non-parametric and multiple comparison by Dunn test ($p < 0.05$).

(750.41, 697.42, 1177.69, 853.72 and 754.00 % at 12, 24, 48, 72 and 96 h, respectively). Mixture exposed organisms presented a similar behavior when compared to control group during the whole experiment. Fig. 2c shows blood results, where a tendency of micronuclei frequency to increase in groups exposed to diclofenac and aluminum is observed at 48 and 96 h. On the other hand, at 12, 24 and 72 h there was a significant increase in diclofenac exposed group (522, 625 and 361 %), while for the aluminum the significant increase occurred at 24 h (400 %). This is particularly noticeable for the diclofenac exposed group at 12 and 24 h. Organisms exposed to the mixture show similar behavior when compared to control.

The percentage of TUNEL-positive cells induced by the different treatments in the liver tissue is shown in Fig. 3a. The group exposed to diclofenac has the highest percentages of TUNEL-positive cells in the experiment, being significant ($p < 0.05$) at 72 and 96 h when compared to control group (382 and 300 %) and the mixture (231.20 and 128.57 %). Groups exposed to aluminum and the mixture did not present noticeable differences when compared to control group along the experiment. Fig. 3b shows results for the TUNEL test on gill cells. The aluminum exposed group showed significant increments ($p < 0.05$) with respect to control at 12 and 72 h (235.33 and 285.77 %). Diclofenac exposed group showed a similar behavior when compared to control group. On the other hand, organisms exposed to the mixture presented a tendency to increase along the experiment, being significant only ($p <$ 0.05) at 48 and 72 h of exposure time (111.10 and 533.35 %). Finally, Fig. 3c present results of the TUNEL test in blood cells. As can be seen, in the group exposed to diclofenac there was an increase in this parameter at all exposure times, being significant at 24, 72 and 96 h (1683.34, 1763.35 and 1914.14 %, respectively). In like manner, the group exposed to aluminum showed significant increases with respect to control at 72 and 96 h (1470.00 and 1521.32 %). The group tested with the mixture behaved similarly to control group, with significant differences when compared to the diclofenac and aluminum tested groups at 48 and 72 h, showing a decrease of about 90 % Fig. 4 shows results for caspase-3 activity in analyzed tissues. Liver tissue (Fig. 4a) showed a significant increase in this parameter in the groups exposed to diclofenac, aluminum and the mixture when compared to the control group at all exposure times, being only significant ($p < 0.05$) for the mixture at 24, 48 and 96 h (804.14, 674.87 and 466.88 %). The organisms exposed to the mixture of both contaminants showed the greatest increase in enzymatic activity with respect to the results of those exposed to the substances individually (126.10, 176.80 and 90.62 % for DFC and 497.33, 367.44 and 79.26 % for Al). The gill tissue of individuals exposed to diclofenac (Fig. 4b) showed no significant increases in caspase enzyme activity throughout the experiment. On the contrary, the group exposed to aluminum showed a significant increase in caspase activity from 12 h and until the end of the experiment (881.75, 1512.35, 236.30, 495.55 and 144.78 % at 12, 24, 48, 72 and 96 h, respectively).

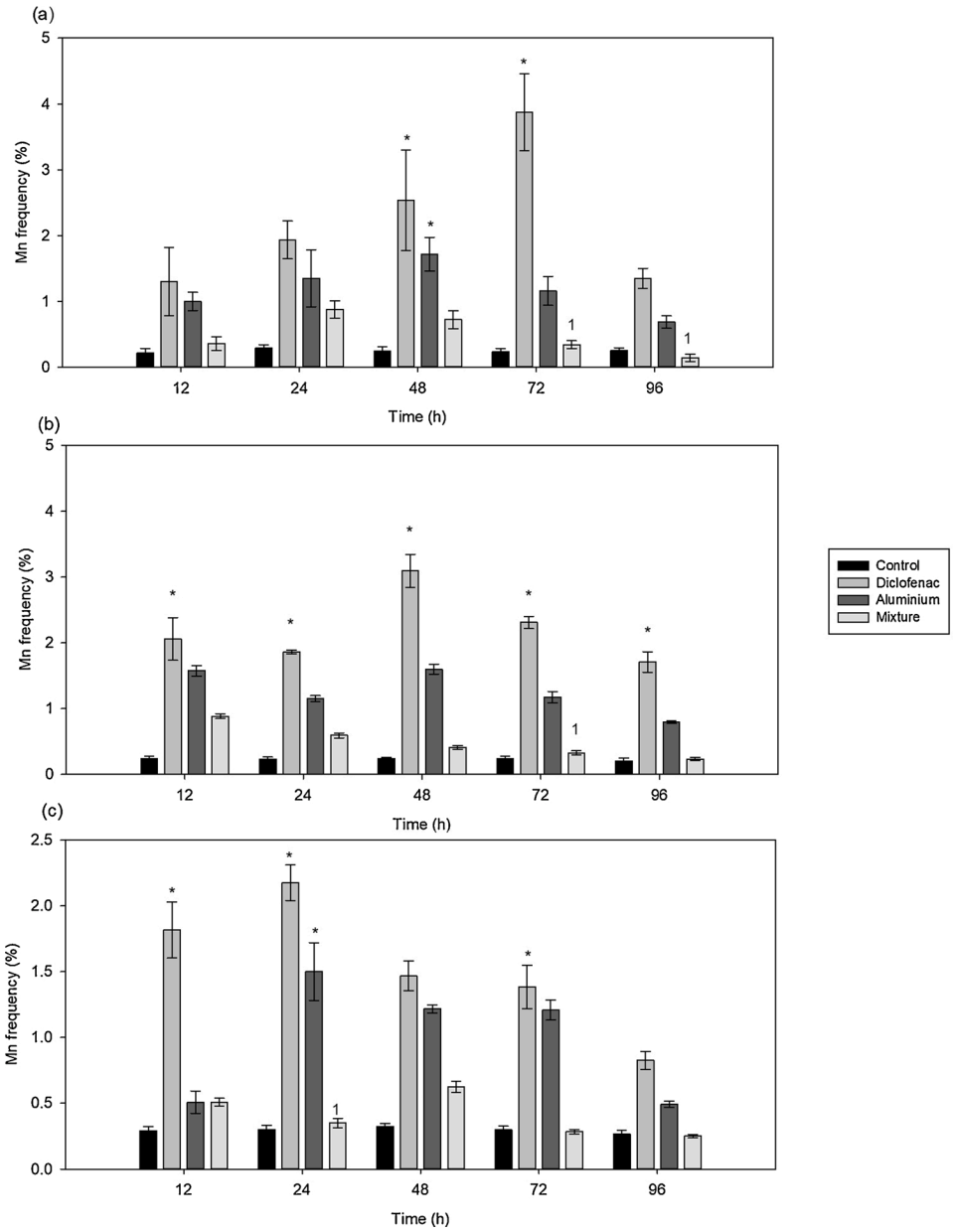


**Fig. 2.** Micronuclei frequency in hepatic (a), gill (b) and blood (c) cells of *Cyprinus carpio* exposed to relevant environmental concentrations of diclofenac, aluminum and their mixture. * Significant statistical difference with respect to the control group; 1) significant statistical difference compared to diclofenac. Statistical test: Kruscal- Wallis non-parametric and multiple comparison by Dunn test ($p < 0.05$).

The organisms exposed to the mixture of xenobiotics showed a tendency to increase with respect to control group at 12, 24, and 96 h of exposure time (98.94, 391.18 and 169.46 %). The effect on blood tissue is shown in Fig. 4c. Diclofenac produces a tendency to increase the caspase activity at 12, 24 and 48 h (88.82, 27.87 and 39.51 %). After that, treatment showed a behavior similar to control group. Aluminum causes important increases in enzyme activity starting at 12 h and until the end of the experiment (200.67, 413.10, 375.38, 360.19 and 368.40 % at 12, 24, 48, 72 and 96 h, respectively). The mixture induces an increase in caspase enzyme activity during all exposure times with respect to control (181.64, 194.72, 220.40, 267.43 and 115.24 % at 12, 24, 48, 72 and 96 h, respectively).

Obtained scores for embryonic development are shown in Fig. 5. Groups exposed to diclofenac and aluminum delay embryos' development starting at 24 h of exposure time and lasting until the end of the experiment. Nevertheless, these delays are not significant with respect to control. On the other hand, the mixture produces a significant delay in embryonic development at 48 ($p$=0.003) and 96 ($p$=0.005) hours of exposure time.

Teratogenic index results are presented in Table 1. Diclofenac treated group showed no important statistical differences with respect to control group, as no malformations were detected through the whole experiment. Regarding the group exposed to aluminum, it showed a statistically different teratogenic index, presenting malformations in the head and in the structure of the cords and presence of pericardial edemas. On the other hand, the mixture of xenobiotics produces a slightly lower teratogenic index when compared to aluminum individually. Nevertheless, such value was statistically different when compared to control group. The types of malformations observed in the group treated with the mixture of contaminants were the same as those found in the group exposed only to aluminum.

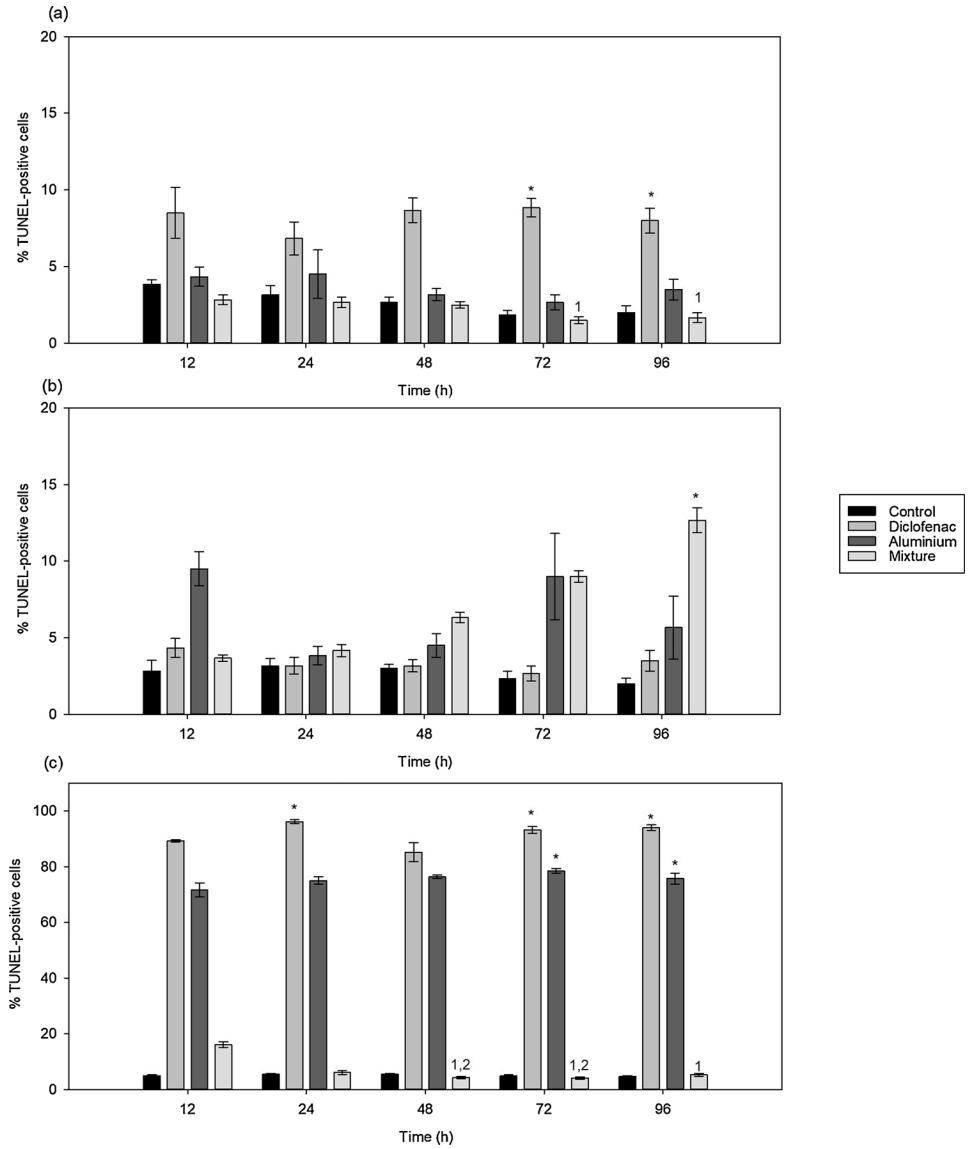


**Fig. 3.** Percentage of TUNEL-positive cells in hepatic (a), gill (b) and blood (c) cells of *Cyprinus carpio* exposed to relevant environmental concentrations of diclofenac, aluminium and their mixture. * Significant statistical difference with respect to the control group; 1) significant statistical difference compared to diclofenac; 2) significant statistical difference with respect to aluminum. Statistical test: Kruscal- Wallis non-parametric and multiple comparison by Dunn test ($p < 0.05$).

## 4. Discussion

The geno and cytotoxic effects as well as the teratogenesis produced by diclofenac, aluminum and their mixture on *Cyprinus carpio*, were evaluated in the present study. In general, the assessment of the risk to the health of aquatic organisms from exposure to this type of contaminant is mainly based on the toxicity data of individual xenobiotics. However, studies of the toxicity of the mixture of aluminum and diclofenac are necessary, in particular with regard to the genotoxic effects due to recent reports of the carcinogenic potential of both compounds and its constant occurrence in aquatic environments (Hong et al., 2007; Ribas et al., 2014; Darbre, 2016; Mandriota et al., 2016; Mandriota, 2017).

The results concerning the damage to DNA by the fragmentation of a strand or the formation of adducts (Fig.1, comet assay) shows that aluminum and the mixture of xenobiotics produces significant increases in the index of DNA damage in blood and liver at different exposure times. It is known that aluminum is capable of establishing bonds or adducts with sulfhydryl groups of enzymes and phosphate groups of nucleic acids. In addition, it is able to displace intracellular iron and through the Fenton reaction, trigger the production of hydroxyl radicals and thus induce DNA nitrogen bases oxidization (García-Medina et al., 2011), which would explain the results obtained, both in the group of organisms exposed to Al and to the mixture of xenobiotics.

In the case of diclofenac, at the concentration tested, a significant increase in DNA damage was observed only at 72 h in liver cells. The nucleophilic groups of deoxyribose and nitrogenous bases of DNA are exposed to the electrophilic attack of reactive oxygen species (ROS) which reach the cell nucleus and are formed because of external agents or cellular metabolic processes (Shi et al., 1996). In this sense, the transformation of diclofenac can generate hydroxylated metabolites such as 5-hydroxydiclofenac, plus free radicals (van Leeuwen et al., 2011), which would explain the damage produced by diclofenac and the mixture of xenobiotics in the liver of common carps. This effect coincides with that observed by Pandey et al. (2017) in hepatic cells of *O. niloticus* exposed to 0.68 mg $L^{-1}$ of diclofenac, who mention that the damage to the DNA found could have developed from DNA-protein cross-links break, DNA double strand break, DNA adducts formations

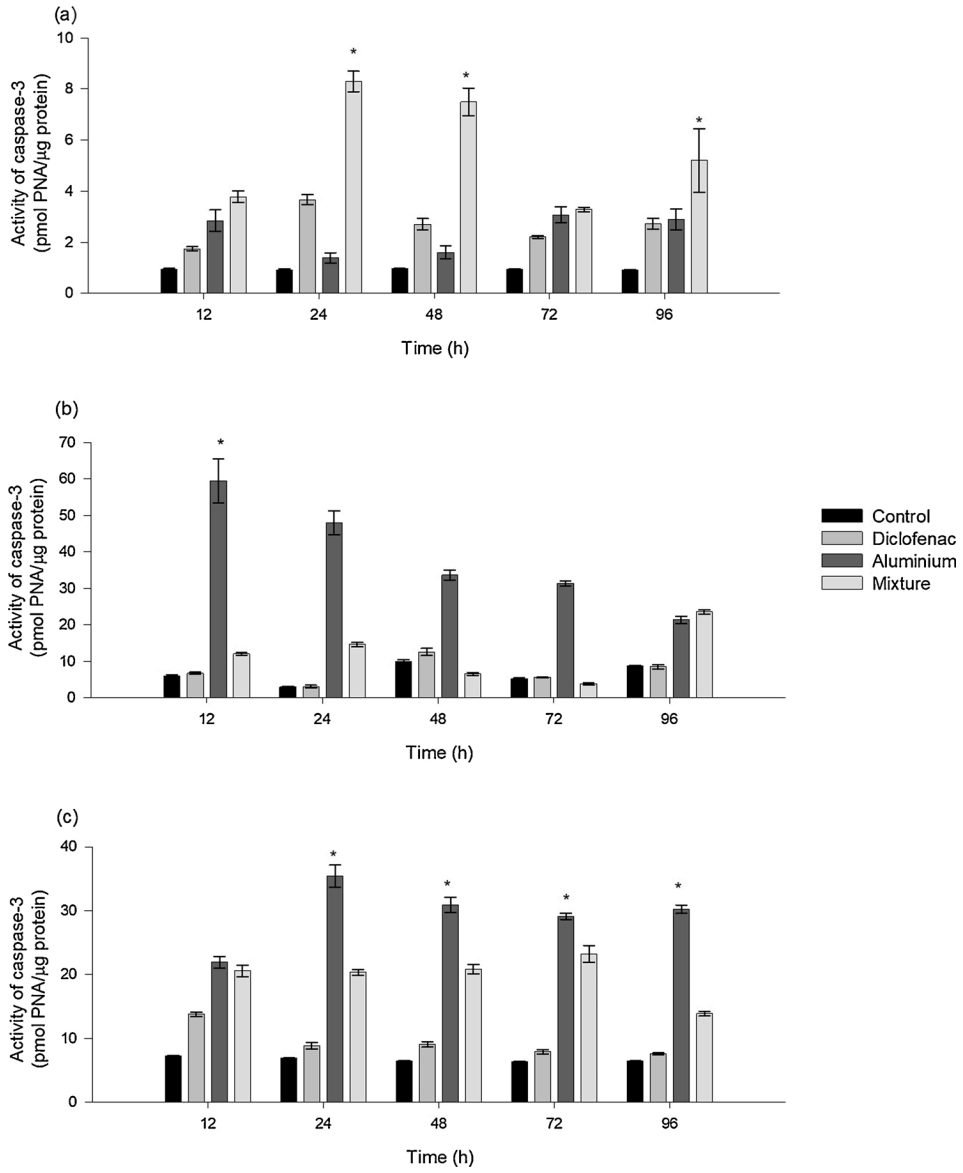


**Fig. 4.** Caspase-3 activity in hepatic (a), gill (b) and blood (c) cells of *Cyprinus carpio* exposed to relevant environmental concentrations of diclofenac, aluminum and their mixture. * Significant statistical difference with respect to the control group. Statistical test: Kruscal- Wallis non-parametric and multiple comparison by Dunn test ($p < 0.05$).

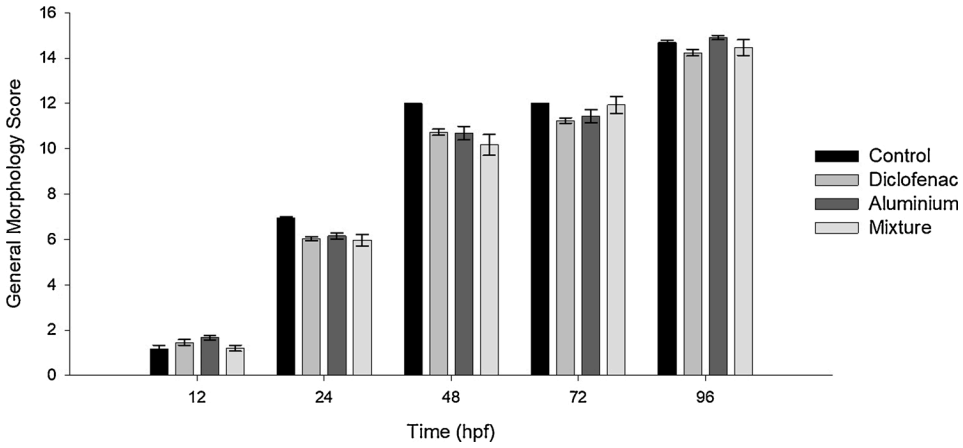


**Fig. 5.** General morphological scoring of *Cyprinus carpio* embryos exposed to environmentally relevant concentrations of diclofenac, aluminum and their mixture. Statistical test: Friedman repeated measures analysis of variance on ranks with a Dunn multiple comparison ($p < 0.05$).

**Table 1**
Teratogenic index produced on *Cyprinus carpio* embryos after exposure to diclofenac, aluminum and their mixture at environmentally relevant concentrations.

| | Control | Diclofenac | Aluminum | Mixture |
|---|---|---|---|---|
| **Mean ± E.S** | ± 0.00 | ± 0.00 | 0.0354 ± 0.00832 [a,b] | 0.0273 ± 0.0119 [a,b] |
| **Malformations frequency per experimental group** | | | | |
| | | | - Head malformations: **16.66%** | - Head malformations: **11.11%** |
| | | | - Cord structure malformations: **11.11%** | - Cord structure malformations: **11.11%** |
| | | | - Pericardial edema: **8.33%** | - Pericardial edema: **5.55%** |

[a] Significant difference with respect to control group.
[b] Statistically significant difference with respect to diclofenac treated group. (Statistical trail: U- Mann- Whitney $p < 0.05$).

and DNA–DNA and DNA single-strand resulting from the interaction between the toxic and its metabolites with DNA.

Regarding to gill cells, none of the treatments shows a genotoxic effect at DNA level. In the case of Al, it has been reported that chlorine cells, found in the primary gill lamellas, serve as storage site for aluminum, since they have shown that after an exposition to this metal of up to 48 h Al is precipitated into the cytoplasm of cells. In addition, it has been observed that under these circumstances cell proliferation increases to replace damaged cells. (Norrgren et al., 1991; Poléo et al., 2017). It is likely that aluminum not stored in chlorine cells remains free. It may later quickly enter systemic circulation and focus its toxic effect in other target sites.

In the blood, an increase in DNA damage caused by aluminum can be observed from the first exposure times, being only significant at 48 h (corresponds to the maximum damage). In the mixture the behaviour is similar, with two significant maximums at 24 and 96 h. The damage is likely to remain constant over time due to the gradual release of aluminum from the gill chloride cells, which release the xenobiotic depending on the concentration of free toxicant. The constant disposition of the xenobiotic generates a damage that with the passing of time can accumulate, presenting a maximum damage time. In response, repair systems can be activated or, if the damage cannot be repaired, cells can experience programmed cell death (Chatterjee and Walker, 2017).

In the case of mixtures of pollutants with different specific modes of action, interactions may occur, and the changes are basically modifications of the toxicity of the pollutants (Gómez-Oliván et al., 2017; Luja-Mondragón et al., 2019; Sanjuan-Reyes et al., 2020), as observed in the different biomarkers evaluated. Thus, fluctuations in the level of damage to DNA observed in the cells of organisms exposed to the mixture of diclofenac and aluminum, have also occurred in mixtures of other contaminants, for example, Stankevičiūtė et al. (2017) observed a significant increase in damage to DNA, at 7 days of exposure in *Salmo salar* erythrocytes exposed to a mixture of metals (Zn, Cu, Ni, Cr, Pb and Cd), the damage was evaluated by the frequency of micronuclei. This assay allows for chromosomal rupture detection caused by an inadequate reparation of DNA molecules during cell cycle or by a direct toxic effect over cellular division mechanisms (Bolognesi and Hayashi, 2011).

In Fig. 2, it can be seen that the group exposed only to diclofenac showed significant increases in most of the exposure times in the three tissues evaluated. As mentioned previously, diclofenac biotransformation and photo-degradation process lead to hydroxylated metabolites formation, such as the 5-hydroxydiclofenac and ROS. These radical species can oxidize the genetic material producing different types of damage, including single or double strand breaks in the sugar-phosphate skeleton, modification of nitrogenous bases (thymine ring saturation and fragmentation), and formation of DNA-protein or DNA-DNA through various mechanisms: modification of DNA bases (OH radical action leads to over 20 modifications, the most common being 8-hydroxy-2′-deoxyguanosine (8-OHdG) which has high mutagenic potential), depurination of DNA bases (apurinic or apyrimidinic sites formed by cleavage of the glycosidic bond, which may result from OH· radical attack on sugar), and strand breaks (due to cleavage of the phosphodiester bond, occurring frequently by free radical attack on deoxyribose of the DNA backbone) (Islas-Flores et al., 2017). This can cause ruptures of adjacent phosphodiester bonds at the site of abstraction at the time of distribution of genetic material in cell division, plus consequent micronuclei formation (Bolognesi and Hayashi, 2011).

Compared to those exposed to diclofenac, no significant chromosomal damage was apparently found in organisms exposed to aluminum. However, in most exposure times non-significant levels were observed above the control group. It is probably that the lesions at the molecular level produced by Al, and evidenced by the comet assay, have been repaired by enzymatic systems or eliminated by apoptosis, so there was no damage at the chromosomal level. On the other hand, aluminum can cause chromosomal damage because of its ability to modify tubulin structure or destabilize lysosomal membranes, which could lead to the release of lysosomal DNAase in the cytoplasm, which would explain the non-significant increases in this parameter (Banasik et al., 2005).

In the case of the organisms exposed to the xenobiotic mixture, no significant effect was shown on the frequency of micronuclei in the tissues studied, these values were lower compared to the groups exposed to diclofenac and aluminum individually. All these results indicate that possibly there was some kind of antagonistic action between diclofenac and aluminum. Antagonism occurs when one or more compounds in a mixture interfere with the effect of another. Consequently, there is a reduction in the predicted effect for individual compounds that do not need to be structurally similar (López-González et al., 2019). There are various mechanisms by which xenobiotics can produce an antagonistic interaction, including when a compound stimulates the biotransformation of a second or interferes in some way with its absorption (Hernández et al., 2013). Diclofenac present in the xenobiotic mixture does not induce the chromosomal genotoxic effect observed in organisms individually exposed to this pollutant. The gills are the main site of absorption of oxygen, nutrients and xenobiotics in the fish. Several studies have shown that some toxics, including Al, produce morphological changes in this organ, including gill hyperplasia and secondary lamellae hypertrophy, which increment gill thickness and diminish its diffusion gradient between blood and the surrounding water (Poléo et al., 2017). This phenomenon explains the remarkable difficulty of the organisms to breathe during the experiment and probably the reduced diffusion of diclofenac into the systemic circulation, and consequently the lower damage produced.

On the other hand, there may also be a reduction in the effects on the frequency of micronuclei due to alteration in the rate of cell renewal, for example, a decrease in erythropoiesis (Udroiu, 2006). Therefore, the low frequency of MN presented in the organisms exposed to the mixture could be due to the alteration of the kinetics of the cells and the accelerated replacement of these and not to a lesser genotoxic effect.

Usually, injuries by chromosomal fracture commonly activate cell death processes by apoptosis. One of the most relevant events in the development of apoptosis is the activation of caspase enzymes, which initiates the apoptotic process. These enzymes lead to endonucleases systems activation (for DNA degradation) and cytoskeleton degradation, which induces apoptotic bodies formation (Choudhary et al., 2015). DNA degradation is one of the most relevant apoptosis characteristics and can be evaluated by the TUNEL test (Mohan et al., 2015).

Fig. 2 shows an increase in TUNEL-positive cells, depending on time,

tissue and tested xenobiotic. Thus, in the blood cells exposed to diclofenac, the increase occurs at 72 and 96 h, which coincides with the decrease in the frequency of MN. Diclofenac may affect red blood cell membranes in many ways. It interacts with a specific class of lipids found in the erythrocytes plasmatic membrane exterior, which induces a disorder effect over the acyl chains of the membrane phospholipid bilayer. This also modifies the biconcave form of red blood cells, which become especially susceptible to cell death by an external membrane permeability modification (Suwalsky et al., 2009).

In the case of liver, there is a significant increase in apoptosis production, particularly in organisms exposed to diclofenac between 24 and 96 h. There are reports that diclofenac activates apoptosis in hepatocytes mainly via mitochondria. Diclofenac biotransformation metabolites (4 and 5-hydroxydiclofenac), together with oxygen reactive species formed in this process, have proved to modify multiply ways of cellular signaling. These include transcription factors, such as the (NF)-E2 nuclear factor that with the 1(Nrf-1) factor participate in proliferation and differentiation processes, as well as cell death by apoptosis (Cardoso--Vera et al., 2017). Studies in mammals have shown that exposure to different diclofenac concentrations cause an early increase in Bcl-xL proteins expression, as well as oxygen reactive species generation. These effects depend on the concentration and occur inside the mitochondria (Gómez-Lechon et al., 2003; Syed et al., 2016; Amanullah et al., 2017).

In the gills there is no increase in TUNEL-positive cells, nor in caspase activity (Fig. 4) in organisms treated with diclofenac, this is consistent with the increased frequency of MN at all exposure times. In contrast, in the gills of organisms exposed to aluminum there was a significant increase in caspase-3 activity at 12 h of exposure, maintaining this tendency until 96 h. The same behavior was observed in the TUNEL test. In freshwater, aluminum labile forms (cationic/inorganic) accumulate in gills, causing a regulatory and respiratory iono- and osmoregulatory dysfunction, that can cause the death of the organisms (Poléo et al., 2017). Failure of the cell to regulate the input and output of ions can eventually increase intracellular calcium levels. On the other hand, aluminum is characterized by cause an increase in reactive oxygen species that compromise antioxidant defenses. Both events promote apoptosis (Mustafa-Rizui et al., 2014).

In the hepatic cells of organisms exposed only to aluminium, an increase in caspase-3 activity is observed between 24 and 96 h. A similar result was observed in the frequency of TUNEL-positive cells in most exposure times, being significant at 72 and 96 h. Aluminum can reduce the availability of ATP for cell consumption, which is associated with increased calcium input into the cells. This increase is associated with the activation of programmed cell death processes, even causing the translocation of phosphatidyl serine to the outside of the cell (Herlax et al., 2011). In addition, Al induces the release of cytochrome c from mitochondria, a decrease in Bcl-2 in both the mitochondria and the endoplasmic reticulum, the translocation of Bax into mitochondria, the activation of caspase-3 and DNA fragmentation (Ghribi et al., 2001).

In the case of the blood cells of organisms exposed to Al, there was a tendency to increase the activity of caspase-3 at 12, 72 and 96 h. $Al^{3+}$ stimulates the translocation of phosphatidyl serine outside the cells, which stimulates cell phagocytosis and reduces cell volume. Several studies have demonstrated that aluminum intoxication induces red blood cells eryptosis (Herlax et al., 2011). This element is capable of affecting pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) proteins, as well as transcription factors such as the p21 and p53. It has also been shown to exert oxidative stress on the lymphocytes of exposed carps, oxidizing proteins involved in the process of cellular cycle regulation, causing an unbalance of this process and increasing cell death by apoptosis (García-Medina et al., 2011).

The mixture of xenobiotics demonstrated to induce the most elevated blood caspase-3 enzymatic activity and TUNEL-positive cells at 72 and 96 h of exposure. A similar effect was observed in the gills at 96 h in the TUNEL test, presenting a possible synergy. A synergistic effect occurs when the effect of exposure to a mixture of xenobiotics is greater than or different from that expected by an additive effect (sum of effects), inducing responses not predicted by their individually known effects. When synergistic effects are observed, one of the xenobiotics changes the response in the organism qualitatively or quantitatively. A quantitative response is one that is much greater than what would be observed for an additive effect and a qualitative effect is the result of attacking different targets and therefore the response cannot be predicted (Zeliger, 2011). Both pollutants, Al and diclofenac, cause cytotoxic effects individually, so cytotoxic activity of the mixture may be the outcome of the individual effects of each xenobiotic.

Stepanova et al. (2013) determined that although diclofenac biotransformation metabolites, along with the free radicals generated in that process, are especially toxic to aquatic organisms at concentrations between 0.015 and 3 mg $L^{-1}$, no negative effects were observed in carp embryos. The concentration employed in the present research is considerably lower and likewise, no negative effects on embryos were observed.

For its part, a study on biphasic vertebrate embryos treated with metallic cations showed that aluminum at a concentration of 1.5 mMol is significantly more toxic than chrome on the *Triturus vulgaris meridianalis*. Al causes malformations that affect the heart system. In addition, the embryos showed blisters and edema on the surface of their bodies, probably due to the ability of this metal to interrupt the transport of metal ions (Calevro et al., 1998).

The Al concentrations used in this study, both individually and in mixture, cause head and cord structure malformations, as well as pericardial edemas. These results coincide with what was observed in zebrafish larvae embryos exposed to 25–100 μM of aluminum where it was shown that the nervous and circulatory systems were the most affected (Monaco et al., 2017). This is probably due to the ability of this metal to interfere with the transport of metal ions across the cell membrane and its ability to indirectly induce reactive oxygen species and free radicals. This compromises the antioxidant defense systems, which are especially important for the embryos, to maintain the REDOX balance and reduce the frequency of the negative effects on the embryonic development.

In conclusion, diclofenac and aluminum at environmentally relevant concentrations, act as genotoxic, cytotoxic, embryo-toxic and teratogenic agents on *C. carpio*. The toxic response obtained when testing the mixture of both xenobiotics is completely different when compared to that of substances individually. This is especially important considering the fact that contaminants in the aquatic environment are not found in isolation, but in complex mixtures, and therefore interactions that affect the expected toxic effects may occur.

## Authorship statement

Manuscript title: Geno-cytotoxicity and congenital malformations produced by relevant environmental concentrations of aluminum, diclofenac and their mixture on Cyprinus carpio. An interactions study.

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in the Environmental Toxicology and Pharmacology journal.

## Authorship contributions

Please indicate the specific contributions made by each author (list the authors' initials followed by their surnames, e.g., Y.L. Cheung). The name of each author must appear at least once in each of the three categories below.

Conception and design of study: M. Galar-Martínez, S. García-Medina;

acquisition of data: E.H. Quiroga-Santos, S. Cano-Viveros, K. Ruíz-Lara, H. Islas-Flores;

analysis and/or interpretation of data: E. Gasca-Pérez, L.M. Gómez-Oliván.

Drafting the manuscript: M. Galar-Martínez, S. García-Medina, E.H. Quiroga-Santos, E. Gasca-Pérez;

revising the manuscript critically for important intellectual content: L.M. Gómez-Oliván, S. Cano-Viveros, K. Ruíz-Lara, H. Islas-Flores.

Approval of the version of the manuscript to be published (the names of all authors must be listed):

E.H. Quiroga-Santos, M. Galar-Martínez, S. García-Medina, E. Gasca-Pérez, S. Cano-Viveros, K. Ruíz-Lara, L.M. Gómez-Oliván, H. Islas-Flores.

## Ethical approval

All applicable international, national and institutional guidelines for the care and use of animals were followed. All procedures were performed in accordance with the ethical standards of the institution where the studies were conducted.

## Declaration of Competing Interest

The authors report no declarations of interest.

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