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Genotoxic and cytotoxic alterations induced by environmentally-relevant concentrations of amoxicillin in blood cells of *Cyprinus carpio*

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HIGHLIGHTS

- Cyto-genotoxicity of AMX was evaluated at 0.039 and 1.65 μg L^{-1}
- AMX was proven cytotoxic with caspase-3 activity and TUNEL test.
- AMX was genotoxic proven with comet assay and micronucleus test.
- The highest cyto-genotoxic effect was found in C1 and C2 at 72 h in *C. carpio*.

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ABSTRACT

Amoxicillin (AMX) is a pharmaceutical widely employed in human and veterinary medicine worldwide. Its wide production and use has led to this pharmaceutical being released into the environment in concentrations that range from ng L^{-1} to μg L^{-1}. Previous studies have demonstrated that this antibiotic generates toxic effects, amongst which alterations to embryonic development and oxidative stress in aquatic organisms, is noteworthy. Nonetheless, it is necessary to characterize the risks that this pharmaceutical represents for species of economic interest such as *Cyprinus carpio*, in a more precise manner. The aim of this work was to demonstrate if AMX, at environmentally-relevant concentrations, is capable of inducing genotoxic/cytotoxic alterations in *C. carpio*. In order to evaluate genotoxicity, the comet assay and micronucleus test were used; in order to determine cytotoxic effects, caspase-3 activity and the TUNEL assay were carried out. The results showed that the effects of the biomarkers had their maximum at 72 h; considering the DNA damage in the comet assay, 0.039 μg L^{-1} resulted in a 29% increase compared to control, and 1.67 μg L^{-1} caused a 40% increase; micronucleus frequency increased by 205% in C1 and by 311% in C2 when compared to control; compared to control, caspase-3 activity increased 262% in C1 and 787% in C2; for the TUNEL assay, DNA fragmentation increased by 86% in C1 and 120% in C2 compared to control. The results showed that environmentally-relevant concentrations, AMX was capable of generating DNA damage and cytotoxic effects in blood cells of the common carp.

1. Introduction

Among the pharmaceutical products of greatest consumption, antibiotics are found, which are used in large quantities worldwide in human medicine as well as veterinary medicine for the

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treatment of microbial infections and to improve the growth and feeding efficiency of the cattle. In consequence, relatively high concentrations of antibiotics can be found in diverse bodies of water (Manzetti and Ghisi, 2014; Zheng et al., 2012).

Previous studies have reported that the most-used antibiotics are those found in the beta-lactam group, which can be divided into penicillin, cephalosporins, monobactams and carbapenems (Hooper, 2001). These antibiotics are of natural origin or semi-synthetic and are characterized by possessing a beta-lactam ring in its structure. They act inhibiting the last stage of the synthesis of the bacterial cell wall, they have a slow bactericidal action, are relatively independent of plasma concentration, present little toxicity in humans and possess a wide therapeutic margin (Hooper, 2001). Within this group, amoxicillin (AMX) can be found, which is prescribed to treat bronchitis, infections (ear, skin, throat and urinary tract), as well as inflammation of the amygdales. In aquiculture, AMX incorporates itself into granulated foods or is applied directly to water for the treatment of streptococcosis, furunculosis, and pasteurelosis (Lalumera et al., 2004).

The wide medical and veterinary use of AMX contributes to its elimination through different water bodies, via municipal, hospital and industrial waste discharge. This can be demonstrated in different reports in which there is literature available concerning its occurrence (Fatta-Kassinos et al., 2011; Watkinson et al., 2007).

For example, (Kasprzyk-Hordern et al., 2007) reported that in hospital and urban effluents, concentrations of this pharmaceutical were of 0.9 and 1.67 $\mu g\,L^{-1}$, respectively; in surface waters, concentrations between 0.039 and 0.0245 $\mu g\,L^{-1}$, were found. Likewise, diverse reports have exhibited the AMX occurrence in diverse water bodies worldwide: in Hong Kong, concentrations from 0.64 to 76 $ng\,L^{-1}$ in waste water treatment plant effluents have been found (Van et al., 2016); in Palermo, Italy, Zuccato et al. (2010) reported concentrations from 1.80 to 4.60 $ng\,L^{-1}$ in waste water treatment plant effluents; In Mallorca Island, Spain, concentrations between 39 and 283 $ng\,L^{-1}$ (Rodriguez-Mozaz et al., 2015) were determined. Also, AMX has been correlated with diverse toxicological effects in aquatic organisms. Among the studies reported, we can mention Oliveira et al. (2013), who demonstrated that short exposure times, in concentrations of AMX ranging from 10 to 900 $mg\,L^{-1}$, induce premature hatching, as well as malformations such as edema, tail deformations in embryos and alterations of the enzymatic activity in embryos and adults of *Danio rerio*. Liu et al. (2015) reported that AMX generated oxidative stress in the photosynthetic cyanobacteria *Microcystis aeruginosa,* increasing the levels of malondialdehyde and the activity of antioxidant enzymes superoxide dismutase (SOD), peroxidase (POD) and glutathione S transferase (GST), as well the content of glutathione (GSH). In contrast, Matozzo et al. (2016) demonstrated that AMX at concentrations of 100, 200 and 400 $\mu g\,L^{-1}$ diminished the activity of SOD and catalase (CAT) in *Ruditapes philippinarum* and *Mytilus galloprovincialis*. In 2017, an investigation carried out by our group demonstrated that AMX at concentrations of 10 $ng\,L^{-1}$, 10 $\mu g\,L^{-1}$ and 10 $mg\,L^{-1}$ generated an increase in the levels of lipoperoxidation (LPX), hydroperoxide content (HPC) and carbonylated protein content (PCC), as well as SOD, CAT and glutathione peroxidase (GPx) in brain, kidney and gills of *Cyprinus carpio* (Elizalde-Velázquez et al., 2017).

The generation of oxidative stress induced by AMX, demonstrated by some authors, can alter the integrity of biomolecules such as lipids, proteins and DNA, as well as being associated with disorders in organisms which could affect fertility, health and the lifecycle of fish, as has been demonstrated by Theodorakis et al. (2000), and also provoking genotoxicity (Anlas and Ustuner, 2016) and cytotoxicity. However, there are some studies that have shown that AMX does not generate genotoxic effects, for example (de Sousa et al., 2019) showed that AMX at concentrations of 5.13, 10.26, 20.52 and 41.05×10^{-3} mM^{-1} in exposures of 8 h was not genotoxic or carcinogenic for *Tradescantia pallida* and *Drosophila melanogaster*. So the controversy obtained in the results of the genotoxic effects, generates the question of knowing if at environmentally relevant concentrations and in aquatic organisms that may be more sensitive and at different times of exposure these effects are generated.

The genotoxicity can be evaluated through the use of different biomarkers such as unicellular electrophoresis in gel (comet assay), which proved to be a useful test in environmental monitoring and genetic ecotoxicology (Bolognesi and Cirillo, 2014), and the micronuclei assay (MN), which has been indicated as one of the most-employed methods for the measurement of chromosome structural and numeric changes in different systems in vitro and in vivo (Bolognesi and Hayashi, 2011). Cytotoxicity can be determined through different biomarkers; however, caspase-3 activity and the TUNEL assay (terminal deoxynucleotidyl transferase dUTP nick end labeling) have shown to be very efficient in the evaluation of cytotoxicity in aquatic organisms (Alak et al., 2019; Formigari et al., 2007; Sun et al., 2019; Wang et al., 2019). Due to the aforementioned, the aim of this study was to evaluate the genotoxic and cytotoxic alterations induced by environmentally-relevant concentrations of AMX in blood cells of the common carp *C. carpio*.

2. Materials and methods

Unless otherwise indicated, all the reagents employed in the experiments were acquired from Sigma-Aldrich (St. Louis, MO).

2.1. Test substances

Trihydrated amoxicillin (CAS number 61336-70-7, >98.0% purity) $C_{16}H_{19}N_3O_5S \cdot 3H_2O$, 365.40 Da, was purchased from Tokyo Chemical Industry Co., LTD.

2.2. Test organisms and their maintenance

In order to carry out the genotoxicity and cytotoxicity tests, *C. carpio* (common carp) was used as a test organism or subject. The organisms were acquired in one of the most important carp-breeding centers of the country (Tiacaque, State of Mexico), where it was guaranteed that the organisms were in good health conditions, as well as not exposed to any substance that could alter its health. Posteriorly, the organisms were transported to the Environmental Toxicology Lab. in polyethylene bags previously provisioned of water and oxygen. In the laboratory, the carps were placed in tanks with a capacity of 160 L for 15 days, at room temperature with normal periods of light and darkness, with continuous aeriation, pH of 7.4 and fed daily with Pedregal Silver™ for fish.

2.3. Experimental design for the genotoxicity and cytotoxicity studies

For the experiment, organisms of 11.39 ± 0.31 cm size and a weight of 25.71 ± 2.8 g, were used, and these were maintained in similar conditions during the acclimatization period. In the toxicity assays, the test organisms were administered with AMX at concentrations of 0.039 $\mu g\,L^{-1}$ (C1) and 1.67 $\mu g\,L^{-1}$ (C2) (Kasprzyk-Hordern et al., 2007). These were selected due to the fact that they were environmentally relevant, identified in urban effluent and surface waters. It is important to mention that according to studies carried out by Gozlan et al. (2013), the AMX present in tap

water is degraded to pH = 6.5 at 3 days with 82.4%, at 6 days with 90.1% already the 16 days with 92.4%. The main degradation metabolite of AMX is amoxicilloic acid (AMA) and this compound has been identified after 72 h (Gozlan et al., 2013). For the cytotoxicity and genotoxicity tests, 15 test systems with 5 organisms in each of them, were employed: 1) five for concentration 1 (0.039 μg L^{-1}), 2) five for concentration 2 (1.67 μg L^{-1}) and 3) five corresponding to the control group (AMX free). Exposure times employed were 12, 24, 48, 72 and 96 h. The study was carried out in pentaplicate. In total, 300 test organisms were used. Once each exposure time had finalized, the organisms were removed from the systems and were placed in a tank that contained xylocaine solution (0.02 mg mL^{-1}), with the finality of anesthetizing the organisms and obtaining blood samples from the caudal vein with a 1-mL hypodermic syringe, previously heparinized. With the blood samples, the genotoxicity and cytotoxicity studies were carried out, employing the methods described as follows:

2.4. Evaluation of genotoxic effects

The genotoxic effects induced by AMX in blood cells of *C. carpio* were evaluated through two biomarkers: 1) single-cell gel (comet assay) and 2) micronuclei test.

2.4.1. Single-cell gel electrophoresis (comet assay)

Genetic damage evaluation by unicellular gel test (*comet assay*) was carried out in five consecutive steps: 1) suspension of lymphocytes in agarose, 2) release of DNA in lysis solution, 3) unicellular electrophoresis, 4) neutralization, and 5) unicellular stain with ethidium bromide. For this purpose, the methodology established by Tice et al. (2000) was used. One day before the test, slides were prepared by placing a 100 μL layer of 1% high melting point agarose. The next day, 10 μL of the lymphocyte suspension was mixed with 75 μL (0.75%) low melting point agarose, and then 75 μL of this mixture were placed on previously prepared slides. For the DNA release, the slides were placed for 1 h at 4 °C in a copplin glass with the lysis solution (2.5 M sodium chloride, 10 mM Trizma, 10% DMSO, 1% Triton and pH 10). The slides were then placed in the electrophoresis chamber under the following conditions: time 20 min, 900 mL cold alkaline solution at 4 °C (300 mM sodium hydroxide and 1 mM EDTA) at pH 13, 300 mA, 25 V and pH > 13. The process was stopped with a neutralization buffer (0.4 M of Trizma base) at a pH 7.4.

Finally, the slides were dried for 6 h, stained with 50 μL of ethidium bromide and examined under the fluorescence microscope (Zeiss) with the program Image-pro plus 5.0 (Media Cybernetics) and a wave filter of 450–490 nm. 100 measurements were made per treatment and the damage was obtained by means of the DI, which is the ratio of the length of the tail of the comet and the nuclear diameter.

2.4.2. Micronuclei test

1000 blood cells were analyzed and the content of micronuclei was determined by the method of Çavaş and Ergene-Gözükara (2005). A drop of blood from the carps (exposed to the different concentrations of environmentally relevant AMX) was placed on a slide; a smear was performed and this was stained with 10% Giemsa stain for 9 min. Micronuclei were observed as DNA fragments in the erythrocytes under an optical microscope.

2.5. Evaluation of cytotoxic effects

The cytotoxic effects induced by AMX in blood cells of *C. carpio* were determined through 1) caspase-3 activity and 2) TUNEL assay.

2.5.1. Caspase-3 activity

2.5.1.1. Cell extract. Positive control consisted of Jurkat cells (ATCC # TIB-152), density of 10^6 cells mL^{-1} and 50 ng mL^{-1} of anti-Fas mAb (clone # CH-11, MBL International, Cat. # SY-001). For the negative control (inhibited apoptosis samples), the same cells and 125 μL of Z-VAD-FMK [carbobenzoxy-valyl-alanyl-aspartyl-(O-methyl) fluoromethylketone] (20 mM) were added. Both solutions were incubated in darkness for 16 h at 37 °C in a humidified atmosphere with 5% CO_2. Cell extracts were obtained by centrifugation at 450 x g at 4 °C for 10 min. Pellet was washed with PBS at 4 °C and resuspended in cell lysis buffer at a concentration of 10^8 cells mL^{-1}. The cells were lysed by freezing-thawing, and incubated on ice for 15 min. The cell lysates were centrifuged at 15, 000 x g at 4 °C for 20 min, finally the supernatant was the cell extract used in the test.

2.5.1.2. Colorimetric assay. The indications established in the kit were followed (CaspACETM, Promega, Madison, WI). In microplates with flat bottom were prepared the following systems:

1) Reaction blank: 32 μL of the caspase buffer [HEPES 312.5 mM, pH 7.5; sucrose 31.25%; CHAPS 0.3125% (3 - [(3-Colamidopropyl) -dimethylammonium] -propane sulfonate)]; 2 μL DMSO; 10 μL dithiothreitol (DTT, 100 mM) and 54 μL of deionized water;
2) Exposed group: 32 μL of caspase buffer; 2 μL of DMSO; 10 μL of DTT, 20 μL of blood and 54 μL of deionized water;
3) Positive and negative control: 32 μL of the caspase buffer; 2 μL of DMSO; 10 μL of DTT; 20 μL of the cell extract (for each case) and 34 μL of deionized water.

To each system, 2 μL of the substrate DEVD-pNA was added. The microplates were covered with parafilm and incubated at 37 °C for 4 h. Finally, the absorbance at 405 nm was read and the specific activity of caspase-3 calculated. The results were expressed as nM of free pNA h^{-1} μg $protein^{-1}$.

2.5.2. TUNEL assay

The ApopTag Fluorescein S7110 kit (Chemicon, Temecula CA) was used to identify apoptotic cells. The kit consists of 6 sequential stages that consisted of: 1) obtaining lymphocytes, 2) cell preservation, 3) cellular fixation, 4) hydration, 5) cell labeling and 6) cell staining. For the first stage, a sample of 300 μL of blood plasma (from carps exposed to environmentally relevant concentrations of AMX) was placed in an eppendorf tube and centrifuged at 3000 g x for 5 min at 4 °C to obtain lymphocytes. These cells were resuspended with 50 μL of mounting solution. A 1 μL of this solution was placed on a slide previously prepared with poly-L-lysine and dried for 5 min at 60 °C. Once the cellular material was dried, it was fixed with cold acetone for 10 min (stage 2). For the third stage, the slides were placed in serial solutions of xylene-absolute alcohol, at 96, 80, 70, 60, 50% (v/v) and finally 30s in distilled water. Hydrated cells were added with 1 μL of proteinase k (20 μg mL^{-1}) for 10 min. Three washes were made with PBS (0.138 M NaCl, 0.0027 M KCl) pH 7.4 for 1 min and 60 μL of the equilibrium buffer was added, followed by an incubation at 37 °C for 1 h, previously adding 65 μL of the TdT enzyme (Stage 4). For cell labeling (Stage 5) a wash with PBS was performed and the anti-FITC conjugate was added at room temperature for 30 min. Finally, for cell staining (step 6), propidium iodide (1.5 μg mL^{-1}) was used as a dye and examined in an epifluorescence microscope with a digital camera.

The negative control consisted of cells treated as described above, but without the addition of TdT, and the positive control consisted of cells treated with DNase I (1 μg mL^{-1}). A total of 100 cells were analyzed per fish and apoptosis was expressed as the percentage of TUNEL positive cells in 100 cells.

2.6. Determination of the concentration of total proteins

Plasma proteins were determined in order to normalize the genotoxicity and cytotoxicity biomarkers evaluated in carps exposed to AMX. For this, 50 μL of plasma and 150 μL of deionized water were placed in a test tube, then 5 mL of Bradford reagent (0.10 g of Coomassie blue, 50 mL of 96% ethanol and 100 mL of H_3PO_4, in 1 L of deionized water). The tube content was homogenized and allowed to rest for 5 min. The absorbance was subsequently read at 595 nm. The data were extrapolated in a standard bovine serum albumin curve. The method used for this determination was Bradford (1976).

2.7. Determination of the concentration of AMX in the blood of Cyprinus carpio

For the determination of AMX in carp blood, the same systems utilized to determine the biomarkers of genotoxicity and cytotoxicity indicated in section 2.3 were employed. From the systems, 5-mL samples of water were taken, to carry out the determination of AMX in water at the different exposure times. For the blood samples, a part was employed in order to carry out the cytotoxicity and genotoxicity studies and the other, in order to carry out the determination of AMX in the blood of *C. carpio*.

For the determination of AMX in water and plasma, a standard solution of AMX (50 μg L^{-1}), using sodium hydroxide in methanol (40 mM) in 50% acetonitrile, was prepared. Different dilutions were prepared from a stock solution in order to carry out the calibration curve and these solutions were stored at 4 °C.

The high-performance liquid chromatography (HPLC)-MS/MS system used was Shimadzu Prominence LC connected to an AB SCIEX API 3200 system. The column used was a C18 Synergi (50 mm × 2 mm, 2.5 μm). The mobile phase was 60:40 formic acid 0.2% and oxalic acid 0.1 mMmixture in 100% of water (A), and 60:40 formic acid 2% and 0.1 mM oxalic acid in 100% acetonitrile (B). Flow rate was 0.3 mL min^{-1}, run time 7.2 min, and injection volume 20 μL. AMX was determined with the above mentioned equipment and electrospray ionization (ESI). The ESI positive mode was used throughout. The MS/MS conditions were ionization TSI, ion spray voltage (IS): positive 35 V, nebulizator (ion source gas 1): 40 psi, dry-gas (ion source gas 2): 60 psi, dry gas temperature: 400 °C, colison gas (CAD): 5 psi, curtain-gas flow (CUR): 30 psi and scan type: positive multiple-reaction mode (+MRM). The limits of detection and quantification were: LOD = 0.11 g ng/L and LOQ = 0.325 ng L^{-1}.

2.8. Correlation between the plasma concentrations of AMX in C. carpio and the biomarkers of genotoxicity and cytotoxicity

A plasma correlation was carried out between both concentrations of AMX: C1 (0.039 μg L^{-1}) and C2 (1.67 μg L^{-1}) and the biomarkers of genotoxicity (comet assay and the presence of MN) and of cytotoxicity (caspase-3 activity and the TUNEL test). Correlation was carried out via a Pearson analysis, employing the Sigmastat v2.03 program.

2.9. Statistical analysis

The normality of the data and homoscedasticity were verified via the Shapiro-Wilk and Levene methods, respectively. The *Bonferroni post hoc* test was applied to evaluate significative differences, with $p < 0.05$. In order to carry out the analysis, the Sigmastat program v2.03 was used.

3. Results

3.1. Single-cell gel (Comet assay)

In Fig. 1, a tendency was observed in the increase of DNA damage in comparison with the control group in the majority of the exposure times for both concentrations. At the amoxicillin concentration of 0.039 μg L^{-1} (C1), a significant increase was identified for exposure times 12, 24, 48, 72 and 96 g with a percentage of 16%, 20%, 22%, 29% and 14%, respectively, with regard to the control group ($p < 0.05$). For the concentration (C2) of 1.67 μg L^{-1} at the times of 12, 24, 48, 72 and 96 h, statistically-significant increases of 25%, 31%, 33%, 40% and 20% were observed with regard to the control group ($p < 0.05$), respectively.

For C1, statistically-significant increases in the DNA damage at the exposure times of 12 and 24 with regard to 72 h, with percentages of 13% and 9%, respectively, were observed. For the same concentration at 96 h, a statistically-significant decrease of 15% in the DNA damage with regard to 72 h ($p < 0.05$) was seen. For C2, a significant increase of 15% DNA damage at 12 h with regard to 72 h

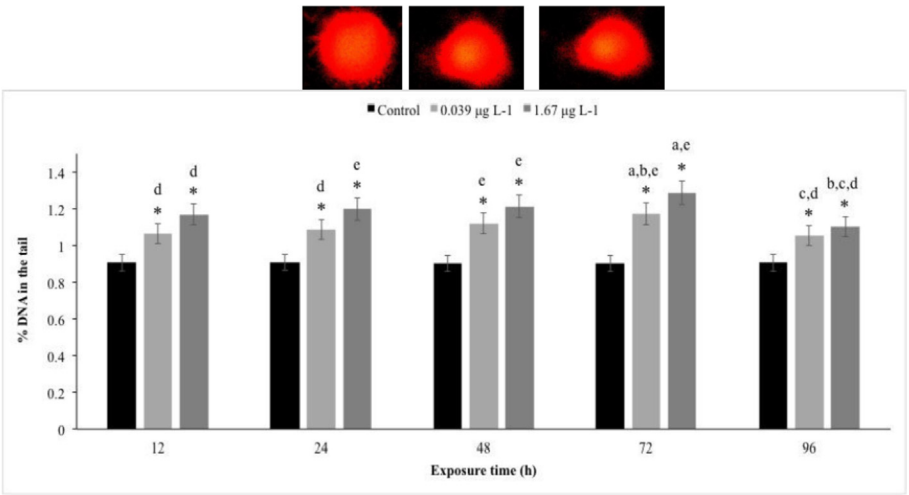

Fig. 1. Determination of the DNA damage via the comet assay in blood cells of *Cyprinus carpio* exposed to AMX. The bars represent the mean ± SEM of the index values of damage of five specimens by concentration and by exposure time. The assay was carried out in pentaplicate. Significantly different from *control group; [a]12 h; [b]24 h; [c]48 h; [d]72 h; [e]96 h (*Bonferroni post hoc*, $p < 0.05$).

and a significant decrease of 20% DNA damage at 72 h with regard to 96 h, was found ($p < 0.05$).

3.2. Micronucleus test

In Fig. 2, a tendency in increase is observed in the MN test in comparison with the control group in the majority of the exposure times for both concentrations. At the AMX concentration of 0.039 μg L^{-1} (C1), a significant increase in the DNA damage was observed for exposure times 12, 24, 48, 72 and 96 h with percentages of 100%, 200%, 150%, 205% and 136%, respectively ($p < 0.05$).

For the concentration of 1.67 μg L^{-1} (C2) at times 12, 24, 48, 72 and 96 h, there was a significant increase of DNA damage with percentages of 60%, 122%, 133%, 311% and 261%, respectively, with regard to the control group ($p < 0.05$).

Comparing exposure times for C1, a significant increase in the DNA damage was observed at 12 h with regard to 24, 48, 72 and 96 h with 100%, 50%, 105% and 36%, respectively. In the case of C2 (1.67 μg L^{-1}), at 72 h, a statistically-significant decrease of DNA damage at 12, 24, 48 and 96 h of 151%, 189%, 178% and 50%, was observed ($p < 0.05$).

3.3. Caspase-3 activity

In Fig. 3, alterations were identified in the activity of caspase-3 with regard to the control group at the majority of the exposure times for both concentrations. A significant increase in the activity of caspase-3 with regard to the positive control for the exposure times of 72 and 96 h was observed, with the percentages of 45% and 59%, respectively in C1 and in a significant decrease of caspase-3 activity down to 62% at 12 h ($p < 0.05$). For C2, a significant increase took place in enzymatic activity of caspase-3 with the percentages of 9%, 59% 110%, 255% and 172%, at 12, 24, 48, 72 and 96 h with regard to the positive control ($p < 0.05$). Comparing the controls with both concentrations, it is observed that for C1, there were significant increases in the caspase-3 activity with the percentages of 58%, 109%, 262% and 250% for 24, 48, 72 and 96 h. In C2, at 12, 24, 48, 72 and 96 h, increases in the caspase-3 activity with the percentages of 155%, 150%, 281%, 787% and 500%, respectively, were observed.

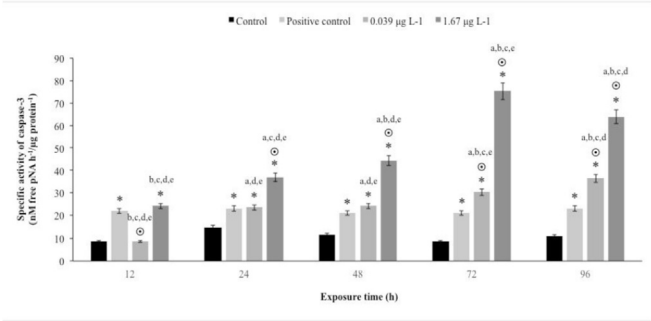

Fig. 3. Caspase-3 activity in blood of *Cyprinus carpio* exposed to AMX for 12, 24, 48, 72 and 96 h. The values are the mean of five repetitions ± SEM. Significantly different from: *control group; ⊙ Positive control; [a]12 h; [b]24 h; [c]48 h; [d]72 h; [e]96 h (*Bonferroni post hoc, $p < 0.05$*).

3.4. TUNEL assay

In Fig. 4, the percentage of TUNEL-positive cells presenting a significant increase in DNA fragmentation with regard to the control group at all the exposure times for both concentrations. For C1, statistically-significant increases in DNA fragmentation with percentages of 28%, 64%, 66%, 86% and 66% with regard to the control group at 12, 24, 48, 72 and 96 h, respectively, were observed. In the same manner, for the same exposure times, increases in DNA fragmentation with the percentages of 57%, 78%, 86%, 120% and 93%, respectively, were observed for C2 with regard to the control.

Comparing both concentrations with regard to the positive control, significant decreases in DNA fragmentation ($p < 0.05$) with the percentages of 75%, 70%, 65%, 64% and 65% were observed for C1 and 69%, 66%, 61%, 57% and 60% for C2, with regard to the positive control at 12, 24, 48, 72 and 96 h, respectively ($p < 0.05$).

3.5. Determination of the concentration of AMX in water and blood of Cyprinus carpio

In Table 1, the concentration data of AMX present in the water of the exposure systems and in plasma of *C. carpio* at the different exposure times employed, are indicated. As can be seen in water after increasing exposure times, a gradual decrease in the concentration of AMX in water and an increase in plasma was

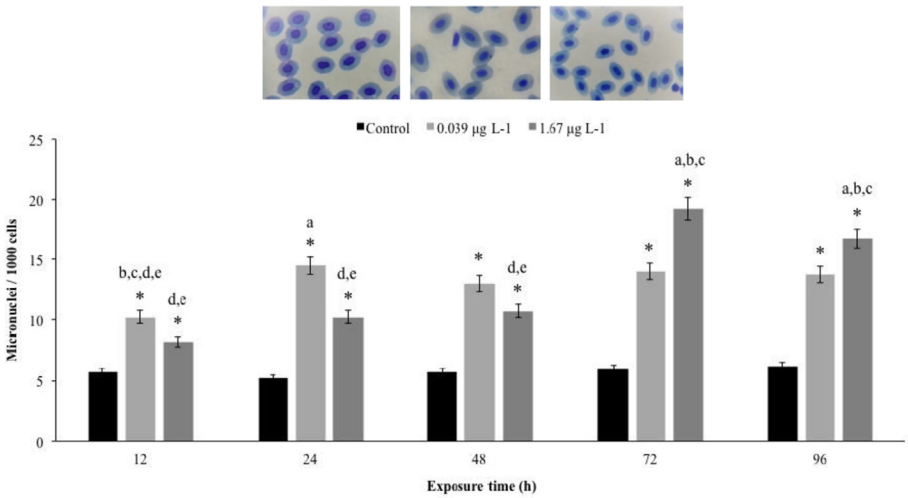

Fig. 2. Frequency of micronuclei in blood of *Cyprinus carpio* exposed to AMX at 12, 24, 48, 72 and 96 h. The values are the mean of five repetitions ± SEM. Significantly different from: *control group; [a]12 h; [b]24 h; [c]48 h; [d]72 h; [e]96 h (*Bonferroni post hoc, $p < 0.05$*).

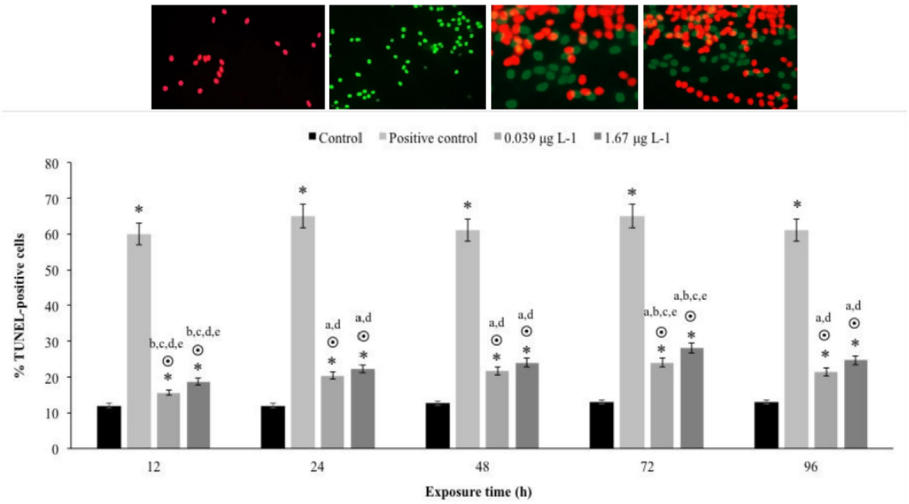

Fig. 4. Determination via TUNEL assay, of the percentage of apoptotic cells in blood of *Cyprinus carpio* exposed to AMX for 12, 24, 48, 72 and 96 h. The values are the mean of five repetitions ± SEM. Significantly different from: * control group; ⊙ Positive control; [a]12 h; [b]24 h; [c]48 h; [d]72 h; [e]96 h (*Bonferroni post hoc, $p < 0.05$*).

Table 1
Kinetics of the concentration of AMX in water in the system and plasma, and bioconcentration factor (BCF) of the common carp *C. carpio*.

Exposure concentration	Exposure time	AMX in water system ng L^{-1}	AMX in blood carp *Cyprinus carpio* ng L^{-1}	Bioconcentration factor (BCF)
Control group	12	ND	ND	0
	24	ND	ND	0
	48	ND	ND	0
	72	ND	ND	0
	96	ND	Nd	0
C1	12	28.1 ± 1.5	8.3 ± 0.8	0.3
0.039 µg L^{-1}	24	22.2 ± 1.3	14.1 ± 1.1	0.6
	48	17.1 ± 1.8	19.3 ± 0.9	1.1
	72	14.3 ± 1.5	22.6 ± 1.2	1.6
	96	12.2 ± 1.8	25.1 ± 1.1	2.0
C2	12	1245.3 ± 2.6	321.3 ± 2.3	0.3
1.67 µg L^{-1}	24	1023 ± 4.1	492.7 ± 3.6	0.5
	48	987 ± 5.2	581.6 ± 4.5	0.6
	72	715 ± 7.3	832 ± 7.3	1.2
	96	523 ± 6.4	992 ± 6.2	1.9

observed. As can be seen for the C1 at 48 h the BCF was greater than 1 and for the C2 at 72 h a BCF greater than 1 was observed.

3.6. Correlation of biomarkers of genotoxicity and cytotoxicity with plasma levels of AMX in common carp C. carpio

In Table 2, the correlation data between BCF of AMX in *C. carpio* and the biomarkers of cyto-genotoxicity observed at different exposure times, are shown. As can be observed, there is a major correlation between BCF of AMX in carp and the biomarkers evaluated in C1: DNA damage, presence of micronuclei and caspase-3 activity were the most relevant; in C2, presence of micronuclei and the presence of apoptosis, evidenced through the TUNEL assay, were those that correlated best.

4. Discussion

This work was carried out due to the fact that, in a previous study carried out by our research team, we demonstrated that AMX was capable of generating oxidative stress at concentrations of 10 ng L^{-1}, 10 µg L^{-1} and 10 mg L^{-1} in brain, gills, liver and kidney of *C. carpio* (Elizalde-Velázquez et al., 2017). Since oxidative stress has been linked to genotoxic and cytotoxic effects in fish (Anlas and

Table 2
Pearson's correlation between BCF of AMX and biomarkers of geno- and cytotoxicity in blood of *C. carpio*.

Biomarkers	Time (h)	AMX	
		0.039 µg L^{-1}	1.67 µg L^{-1}
Comet assay	12	−0.944	0.769
	24	−0.900	−0.990
	48	0.969	−0.035
	72	−0.472	−0.064
	96	−0.490	0.911
Micronucleus test	12	−0.240	−0.654
	24	0.240	−0.500
	48	1.000	−0.740
	72	0.970	0.740
	96	−1.000	−0.981
Caspase-3 activity	12	0.895	0.417
	24	−0.500	0.938
	48	0.500	−0.176
	72	1.000	−0.424
	96	1.000	−0.038
TUNEL assay	12	0.993	0.327
	24	0.327	0.397
	48	0.654	0.500
	72	−0.500	0.500
	96	−0.327	0.327

Correlation coefficients >0.5 are significant (shown in bold).

Ustuner, 2016; García-Medina et al., 2013; Oliveira et al., 2013), we desired to determine if AMX, at environmentally-relevant concentrations, reported in literature worldwide, was capable of inducing cytotoxic and genotoxic alterations in the blood cells of *C. carpio*.

There are some studies that have demonstrated the capacity of AMX of generating DNA alterations, genotoxicity and cytotoxicity in mammals (Arabski et al., 2005; Fahmy et al., 2017; Istifli and Topaktas, 2009; Li et al., 2007); however, some of the authors have demonstrated that the damage to DNA could start at 10 min (Li et al., 2007), but that at 60 min or a few hours later, the reparation processes diminishes the damage. With regard to the genotoxic effects induced by AMX in aquatic organisms, few are the studies which have associated the presence of AMX with damage or alterations to DNA and have been carried out employing very high concentrations of AMX, which are not environmentally relevant. Anlas and Ustuner (2016) demonstrated that at concentrations of 320 mg kg^{-1}, AMX was capable of generating an increase in the frequency of micronuclei and damage to DNA, employing the comet assay in *Oncorhynchus mykiss*. These authors attribute the effect to oxidative stress and suggest that the increase in reactive oxygen species (ROS) could result in the phenomenon of apoptosis. On the other hand, (Oliveira et al., 2013) demonstrated that AMX, at concentrations of 75, 128 y 221 mg L^{-1}, induced oxidative stress and alterations in the hatching rate of embryos and the adults of zebrafish.

These detail that in adults, there was a significant decrease in catalase activity and an induction of the glutathione S transferase enzyme.

As can be seen in the results obtained in this study, AMX at concentrations of 0.039 μg L^{-1} and 1.67 μg L^{-1} was capable of inducing DNA damage, demonstrated via the comet assay and micronucleus test. With the first biomarker, statistically-significant increases of up to 30% in C1 and 40% in C2 were observed; with the micronuclei test, increases of up to 205% for C1 and 311% for C2 were observed. These results are consistent with those obtained by Anlas and Ustuner (2016); the difference in this study is that much lower and thus environmentally-relevant concentrations were employed. Results obtained with both biomarkers could be attributed to the phenomenon of oxidative stress. As we mentioned at the beginning of this section, in the previous study carried out by our research team, we demonstrated that AMX was capable of inducing oxidative stress at low concentrations (10 ng L^{-1} and 10 μg L^{-1}). The reactive oxygen species were capable of generating modifications over purine and pyrimidine DNA bases, on species such as 8-oxoguanine (8-oxoG), thymine glycol and 5-hydroxymethyluracile.

The damage to 8-oxoG and no adequate reparation generates DNA single- or double-strand breaks and mutations in the GC-TA bases, which could lead to genomic instability (Grollman and Moriya, 1993).

As has been previously demonstrated, AMX could induce oxidative stress in diverse species of fish as *C. carpio* and *Danio rerio* (Elizalde-Velázquez et al., 2017; Oliveira et al., 2013); there is a great probability that 8-oxoG is accumulated in the telomers by the elevated incidence of guanine residues in the telomeric DNA sequences (Coluzzi et al., 2014). Likewise, the telomers are repaired with less efficiency than the rest of the genome. Also, 8-oxoG inhibits the activity of telomerase and diminishes the union of the telomeric proteins to the sequence of the telomers, which leads to telomere shortening, the maintenance and the function of the telomers (Coluzzi et al., 2014; Houben et al., 2008; von Zglinicki et al., 2000). It is well-known that oxidative stress induces breaks in only one strand in telomeric DNA. The findings mentioned above could explain our results obtained in the comet assay. On another hand, the frequency of micronuclei was considerably increased in the present work; the elevation of the biomarker could be due to the formation of DNA strand breaks or the lack of the segregation of the chromosomes, the resulting events of the aneugenic and clastogenic effects provoked by ROS (Canistro et al., 2012; SanJuan-Reyes et al., 2015).

One of the main findings found in this study was that AMX at environmentally relevant concentrations was able to generate genotoxic effects. Although the comet assay is more sensitive than the micronucleus test, our results show a contrary effect. The comet assay can detect a wide variety of DNA lesions, making it a more sensitive test, in addition, to evaluate the repair processes of cells. However, it is also well known that free radicals have the ability to generate a micronucleogenic effect by causing breaks in the DNA strand (clastogenic effect) and measuring structural and numerical chromosomal changes in different systems in vitro and in vivo. There are studies that have shown that AMX can generate reactive oxygen species and free radicals after 30 min of exposure in mammalian cells (Li et al., 2007). The genotoxic response depends on the species under study, for example studies carried out by Majone et al. (1988) and (Dixon, 2002) showed that there is a greater response in the micronucleus test with respect to the comet assay in *Mytilus galloprovincialis* due to exposure to mitomycin C that is capable of generating oxidative stress. These authors explain that apparently the increases in the concentration and time of exposure to mutagenic agents would be inhibiting mitotic activity. This allows us to infer that the genotoxic effects can vary according to the physiological characteristics of the organisms, factors such as age, the growth of the organism and the hormonal state, the important influence and the enzymatic systems of activation and genotoxic detoxification.

In their studies, Anlas and Ustuner (2016) mention that the increment of micronuclei and DNA damage could be provoked by the production of ROS and lead to cell apoptosis; however, this hypothesis is not proven by those authors. In our results, this finding could be proven using two biomarkers. 1) the activity of caspase-3 and the TUNEL assay. Apoptosis is programmed cell death and serves the cells as a mechanism in order to maintain homeostasis; The start of this process could be the event that generates cytotoxicity and diverse illnesses (Köhler et al., 2002). There are studies that demonstrate that elevated concentrations of ROS, as well as H_2O_2 and the superoxide radical anion, could lead to the oxidation of the caspase-3 or could also generate a failure over the production of energy on behalf of the mitochondria, which results in cell damage (Asada et al., 2001; Espe et al., 2015; Saucedo-Vence et al., 2017; Yuan et al., 2003).

The mitochondria carry out a fundamental role in the activation of apoptosis induced by multiple stimuli, including the chemical, as in the presence of AMX. It is well-known that mitochondria are a target of ROS (Makpol et al., 2012).

Caspase-3 is an effector in both routes of apoptosis (intrinsic and extrinsic) (Reyes-Becerril et al., 2018). The increase in this biomarker found in *C. carpio* could also be explained by the fact that the reactive oxygen species induced by the exposition to AMX induce a change in membrane potential with the consequent decrease in the production of ATP and an increase in the levels of calcium (Yamamoto et al., 2002; Zhou et al., 2019). The alterations to DNA and an increase of cytosolic calcium initiate the intrinsic route. In this route, cytochrome c and other apoptogenic proteins in the cytosol are released through the aperture of the mitochondrial pores regulated by the Bcl-2 family; all these phenomena are caused by the presence of ROS. Free cytoxhrome c bonds to Apaf1 (activation factor of apoptotic protease 1) forming a multimeric

complex which reclutes and activates procaspase-9 (Gómez-Oliván et al., 2017; Verleih et al., 2019). Activated caspase-9 promotes the activation of the proscaspase-3 effector. Caspase-3 is responsible for the proteolytic cleavage of a wide range of cell targets and leads to cell death (Jin et al., 2019); the increase in the activity of caspase is observed in the apoptotic processes. The apoptosis observed by exposure to AMX in *C. carpio* was confirmed by the TUNEL assay; the results obtained demonstrated a significant increase in positive apoptotic cells. The TUNEL assay is a very efficient biomarker, which is currently employed to detect DNA damage *in situ*; this method has the capacity of identifying in the cell, the initial stages of the apoptotic process, besides those in which morphological changes have been generated, which are apoptotic bodies (Chen et al., 2018; Quan et al., 2019). In this case, cells treated with DNase I were used as a positive control; DNase is an endonuclease which has the capacity of inducing DNA fragmentation (Fahmi et al., 2017; Zhdanov et al., 2015), for which, when compared to the control group, statistically-significant increases were observed; however, when the positive control was compared to C1 and C2, significant decreases between 57 and 75% were observed, but both concentrations showed statistically-significant increases compared to the control group, evidenced by positive apoptotic cells. Results obtained in this biomarker could be explained by the alterations of DNA produced by ROS, induced by AMX in *C. carpio* and also confirm the apoptosis identified with the rise in caspase-3 activity.

Results obtained with the biomarkers as an increase in the activity of caspase-3 and the positive apoptotic cells induced by AMX in the blood of common carp confirm the cytotoxic effects of this antibiotic.

When determining the BCF, we can observe that for C1 there is a higher BCF at 48 h and this is increasing dependent concentration until 96 h. For the C2 the BCF greater than 1 are presented after 72 h and reach the maximum at 96 h. These findings show that AMX is able to bioconcentrate in *C. carpio* blood, and are directly related to the genotoxic and cytotoxic responses identified in this study. However, Gozlan et al. (2013), state that after 72 h, AMX main metabolite (AMA) is formed, so the toxic effects identified can be explained by AMX and AMA. There are few studies carried out on BCF of AMX, for example (Azanu et al., 2016), determine that the BCF for *Daucus corota* L. was 0.4 and for *Lactuca sativa* L., 0.3. This would show that AMX does not have the capacity to bioconcentrate. However, we must consider that these are plant species and the behavior in animals may be different. The findings obtained in this study show that as time goes by, the concentration of AMX in the blood increases. However, we must consider that after 3 days, the main metabolite of AMX is formed.

Pearson correlation between BCF of AMX in *C. carpio* and the biomarkers of geno- and cytotoxicity evaluated showed that in both concentrations, there is a relationship between BCF of the antibiotic and damage to DNA, and the apoptosis presented in blood cells. The maximum responses of the biomarkers of genotoxicity and cytotoxicity are observed at 72 h, and after 96 h, decreases in biomarkers were observed. This evidences that the reparation mechanisms in fish are much more inefficient than those presented in mammals. However, more measurements of the responses evaluated in longer exposure times are required.

5. Conclusions

AMX in environmentally-relevant concentrations, 0.039 $\mu g\ L^{-1}$y 1.67 $\mu g\ L^{-1}$, was capable of generating genotoxic alterations and cytotoxic effects in blood cells of common carp *C. carpio*. AMX, released in a constant manner to the water bodies, represents a risk for the species of fish of economic interest such as *C. carpio*. The biomarkers employed could be of utility in order to demonstrate the effects of AMX in aquatic species.

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