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Original Article

Evaluation of Embryo-larval Toxicity of the Antibiotic Ceftriaxone in the Fish *Danio rerio* (Zebrafish)

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Abstract

Antibiotics comprise a class of pharmaceutical products that have been generating increasing global concern due to their detection in environmental matrices. Cephalosporins constitute the largest group of antibiotics employed for human and animal treatment in most countries. Ceftriaxone belongs to the class of third-generation cephalosporins and can reach the aquatic environment. Studies in the literature have shown the toxic effect of ceftriaxone on zebrafish at high concentrations, and on cyanobacteria at environmental concentrations. The aim of this study was to evaluate the effects of ceftriaxone (0.05 mg/L, 0.5 mg/L, 5.0 mg/L, 50 mg/L and 100 mg/L) on the development of embryos and larvae of zebrafish. The Fish Embryotoxicity Test (FET) was performed according to OECD protocol n. 236. The results showed that the tested concentrations of ceftriaxone did not cause lethal or sublethal effects on zebrafish embryos and larvae. However, considering data in the literature, added to the fact that cephalosporin-class antibiotics are widely used, the use and disposal of ceftriaxone cannot be neglected.

Keywords: Ecotoxicology, Contaminants, Cephalosporins, Environment.

INTRODUCTION

Pharmaceutical products for human and veterinary use are released in the aquatic environment and have been recognized as contaminants of emerging concern (Yan *et al*., 2010; Bojoraski *et al*., 2020). Drugs are long-lasting substances that biodegrade very little. After intake, significant quantities of the original drug and its metabolic residuals are excreted in human and animal urine and feces, arriving in the sewers and effluents of Sewage Treatment Stations, sometimes through domestic sewers, but mainly via hospital sewers (Zapparoli *et al.,* 2011). Pharmaceutical compounds can be found in natural water systems due to disposal by pharmaceutical industries and to their use for treatments in humans and animals (Zapparoli *et al.,* 2011; Feier *et al*., 2018).

Since pharmaceutical compounds are designed to be bioactive even at low doses, this can pose an ecotoxicological threat to aquatic organisms even at environmental concentrations, typically ranging from ng to µg per liter (Caracciolo *et al*., 2015). Antibiotics are among the classes of pharmaceutical products that have been generating increasing global concern, due to the development of antibiotic resistance and because they have been detected in environmental matrices, indicating their ineffective removal from water and wastewater (Das *et al.,* 2019).

A widely used class of antibiotics is the β -lactams, which includes numerous compounds and has a broad antibacterial spectrum (Bush and Bradford, 2016; Bojoraski *et al*., 2020). β -lactams act by disrupting the synthesis of the peptidoglycan layer in the cell walls of

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Gram-positive and Gram-negative bacteria (Magdaleno *et al*., 2015). Cephalosporins represent an important group of antibiotics that belong to the broad class of β-lactams; they constitute the largest portion of antibiotics employed for human and animal treatment in most countries, accounting for 50–70% of use (Das *et al*., 2019).

Ceftriaxone belongs to the class of third-generation cephalosporins and has broader and stronger gram-negative coverage than first or second-generation cephalosporins, in addition to having a long half-life compared to other cephalosporins (Richards *et al*., 1984; Jasprica *et al*., 2023). It has some specific capabilities, such as passing through the cerebrospinal fluid, providing good efficacy against meningitis in children, and the ability to cross the blood-brain barrier in healthy dogs as well as those affected by meningitis (Ringger *et al*., 1996). Ceftriaxone is administered parenterally and is used to treat bacterial infections of the respiratory tract, skin, soft tissues and urinary tract (Richards *et al*., 1984; Jasprica *et al*., 2023). Regarding the physicochemical characteristics, ceftriaxone sodium is a white to yellowish crystalline powder and readily soluble in water (40 g/100 mL at 25 ºC) (Owens *et al*., 2003).

Hernández Martínez *et al.,* (2016) evaluated the toxicity of antibacterials released by a hospital on the Bélico River, Cuba. In their research, the authors analyzed the hospital's consumption of antibiotics for a year and, using a mathematical model, they were able to predict the environmental concentrations of these drugs. According to the study, the most consumed antibiotic was ceftriaxone, with a Predicted Environmental Concentration of 6.2706 µg/L.

Magdaleno *et al* (2014) investigated toxicity of wastewaters from a hospital in Buenos Aires (Argentina). In their research, the authors estimated the quantity of pharmaceuticals used by the hospital during a period of four months and calculated the theoretical antibiotic concentrations (per day) in the hospital wastewater. They calculated the concentration of ceftriaxone at 3.339 mg/L.

The presence of pharmaceutical residues in the aquatic environment presents an important risk to aquatic biology (Magdaleno *et al.,* 2014; Hernández Martínez *et al.,* 2016). Zebrafish have been shown to present a sensitive bioindicator in the assessment of toxicants in the aquatic environment (Fonseca, 2018). Just as the placental transfer of chemical agents occurs in mammals, in their initial stage of life fish can be affected by the transfer of substances through absorption from water by the chorion. This characteristic makes zebrafish a good model for toxicological studies and for biomonitoring pollutants (Fonseca, 2018).

Zhang *et al* (2013) evaluated the toxic functional groups of cephalosporins. These authors tested the toxicity of five cephalosporins (cefaclor, cefoperazone, cefotaxime, ceftriaxone and cefepime) at high concentrations in zebrafish embryos from 6 hpf to 72 hpf. They concluded that the functional groups can induce toxicity in zebrafish embryos, mainly in the development of the cranial nerve, cardiovascular system, notochord and abdomen, and pigment formation.

Zhang *et al.,* (2015) analyzed four antibiotics, including two cephalosporins (ceftazidime and cefotaxime) in zebrafish embryos. Zebrafish exposed to ceftazidine at 6 – 72 hours post-fertilization (hpf) presented abnormal embryos (abnormal abdomens, mild blood pooling and congestion and bent, short bodies) at 15 mg/mL, and zebrafish exposed to Cefotaxime presented abnormal embryos (swollen abdomens and pericardial sacs, deformed heart structures with reduced and congested blood pooling, yolk invagination and short body length) at 10 mg/mL.

Regarding the analysis of environmental concentrations of cephalosporins in bioindicators, Dias *et. al.,* (2015) evaluated the susceptibility of four cyanobacterial isolates to distinct antibiotics, including ceftazidime and ceftriaxone. Ceftadizime inhibited the growth of *Microcystis aeruginosa*, *Aphanizomenon gracile*, *Chrisosporum berghii* and *Planktotjrix agardhii* at concentrations of 0.1 mg/L, 0.012 mg/L, 0.05 mg/L and 0.1–0.2 mg/L, respectively. Cefitriaxone inhibited the growth of these cyanobacteria at concentrations of 0.2 mg/L, 0.006 mg/L, 0.025 mg/L and 0.1–0.2 mg/L, respectively.

Since trace amounts of antibiotics in the aquatic system present a challenge for the assessment of water quality due to their toxic effect on non-target organisms (Vasiliadou *et al*., 2018; Das *et al*., 2019), and there are no studies of environmental concentrations of ceftriaxone in zebrafish, this study aimed to analyze ceftriaxone, a widely used cephalosporin, in zebrafish embryos. The tested concentrations ranged from µg/L to mg/L, considering the worst scenario for antibiotic contamination in the aquatic environment and higher concentrations, up to maximum of 100 mg/L, which is the test limit concentration as recommended by OECD 236, a guideline for testing chemical substances, from the Organization for Economic Cooperation and Development (OECD) (OECD, 2013).

MATERIALS AND METHODS

Drug, administration regime and concentrations

The drug used was ceftriaxone in the commercial form, under the name TRIAXTON®. Each vial of Triaxton® 1000 mg contains 1192.97 mg ceftriaxone disodium hemieptahydrate, which is equivalent to 1000 mg of ceftriaxone base (Triaxton®). The solubility of ceftriaxone in water at room temperature (25 ºC) is 40g/100mL (Owens *et al*., 2003). Ceftriaxone formulated as a solution preparation in sterile water (100 mg/mL) remains stable for three days at room temperature, and remains stable for ten days refrigerated (Owens *et al*., 2003).

The administration regime in zebrafish embryos was through exposure to dilutions of Triaxton® in the water of

the fish culture system according to the Fish Embryo Acute Toxicity Test (FET) described in the guidelines for testing chemical substances (OECD n. 236), from the Organization for Economic Cooperation and Development (OECD). The absorption of the antibiotic by zebrafish was not chemically analyzed. The medium containing the antibiotic was not changed during the FET, which lasted for four days of exposure (96h).

Ceftriaxone concentrations tested were 0.05 mg/L (which corresponds to 50 µg/L), 0.5 mg/L (which corresponds to 500 µg/L), 5.0 mg/L, 50 mg/L and 100 mg/L. The tested concentrations of ceftriaxone consisted of serial dilutions up to 50 mg/L, which allowed a range from µg/L (order of magnitude of concentrations found in the environment) to mg/L (a greater order of magnitude in order to verify toxicity at these concentrations). The maximum concentration tested was 100 mg/L (limit test concentration according to OECD guideline 236).

Fish Embryo Acute Toxicity Test (FET)

Zebrafish (*Danio rerio*) embryos were provided by the Toxicological Genetics laboratory of the Department of Genetics and Morphology at the University of Brasília. The cultivation and maintenance of the fish took place in a specific facility for zebrafish, with water recirculation by ZebTech (Tecniplast, Italy). The fish were kept in aquariums with water filtered by reverse osmosis and activated carbon filters. Temperature remained constantly at 26.0 ± 1 C, ammonia <0.01 mg/L, conductivity at 750 ± 50 mS/cm, pH at 7.5 ± 0.5 and dissolved oxygen at or above 95% saturation. The fish were maintained in a 12:12 h (light:dark) photoperiod cycle.

Adult fish were not used in the experiment, except for the production of embryos. The zebrafish embryos, which were used in the experiment, were obtained from external fertilization in the spawn collection system Ispawn (Tecniplast, Italy). On the day before breeding, males and females were sequentially added to the equipment and kept separated by a divider, in a ratio of two males to one female. Early in the morning, the divider was removed to initiate spawning and fertilization. Embryos were collected immediately after natural fertilization, rinsed in water and checked for viability under a stereomicroscope (Stereoscopic Zoom Microscope and Stemi 2000, Zeiss, Germany). Unfertilized eggs (<20%) and those embryos with cleavage irregularities or lesions were discarded. Embryos not used for the experiments were kept in the vivarium for breeding.

The toxicity test of fish embryos was carried out in accordance with the recommendations of the Organization for Economic Cooperation and Development (OECD), as recommended in its guidelines for testing chemical substances (OECD n. 236). The project received its approval from the Ethics Committee on Animal Use (CEUA) from the University of Brasília, under protocol number 022/2020.

Zebrafish embryos were exposed to the positive control (3.4 dichloroaniline, 4 mg/L), negative control (water from the fish culture system only), and ceftriaxone samples (0.05 mg/L, 0.5 mg/L, 5.0 mg/L, 50 mg/L and 100 mg/L). The test was performed with 72 embryos per exposure, divided into 3 replicates in 24-well microplates (1 embryo per well of the plate). Therefore, for each control or each concentration tested, a total of 3 plates were used. For each plate, a total of 20 wells were filled with 2 mL of the sample to be exposed (positive control, negative control, or tested concentration). The remaining 4 wells on each plate comprised the internal plate control and were filled with 2 mL of water from the fish's own system (as required by the OECD guideline). The test started immediately after fertilization and continued for 96 hours in a climatic chamber, at 27 ºC (SL-24 Solab Científica, Brazil). Embryos and larvae were observed daily under a stereomicroscope for 96 hours post-fertilization (96 hpf). Developmental parameters were used to evaluate the embryos during the test period, using a magnification of 70X for embryos and 40X for hatched embryos (larvae). The parameters of lethality were evaluated according to OECD protocol n. 236 (OECD 2013), which are: coagulation of fertilized eggs, lack of somite formation, lack of detachment of the tail-bud from the yolk sac, and lack of heartbeat. At the end of the exposure period, acute toxicity was determined based on a positive outcome and the LC_{50} was calculated. OECD guideline 236 also mentions that a limit test may be performed at 100 mg/L of the chemical under study, in order to demonstrate that the LC_{50} is higher than this concentration.

In addition to lethality parameters, FET can also indicate changes that precede the individual's death, such sublethality parameters (poor heartbeat, lack of pigmentation, edema) and teratogenic parameters (malformation of head, malformation of tail, deformity of yolk) (Nagel, 2002).

The distinction between normal and abnormal embryonic development was followed according to the description of *Danio rerio* embryogenesis published by Kimmel *et al* (1995). The negative control consisted only of water from the fish culture system, and the positive control was a solution of 3.4 dichloroaniline, 4 mg/L in culture water.

STATISTICAL ANALYSIS

The one-way ANOVA test was used to detect differences between groups for normally distributed data sets. When the data did not pass the Kolmogorov Smirnov test for normality and the Levene test for homogeneity of variance, the Kruskal Wallis test was used. If significant results were found, Dunnett's or Dunn's test (for parametric or non-parametric tests, respectively) was used to detect significant differences between the groups ($p < 0.05$). The Sigmaplot 12.5 statistical package (Systat, nd) was used.

RESULTS

In the toxicity analyses in *Danio rerio* embryos exposed to concentrations of 0.05 mg/L, 0.5 mg/L, 5.0 mg/L, 50 mg/L and 100 mg/L of ceftriaxone, no toxic effects were observed (lethality, sub-lethality or teratogenicity). Embryo development occurred physiologically at all tested antibiotic concentrations.

After analysis, statistically significant differences ($p < 0.05$) were observed only between the positive control and the negative control after 48h (Kruskal Wallis $p = 0.021$; Dunnett's $p < 0.05$), 72h (Kruskal Wallis $p = 0.021$; Dunnett's $p < 0.05$) and 96 h (Kruskal Wallis $p = 0.021$; Dunnett's $p < 0.05$) of exposure.

Figure 1 shows zebrafish embryos/larvae exposed to positive control, negative control, and tested concentrations of ceftriaxone. The images in Figure 1 (A1 – A4) show zebrafish embryos/larvae exposed to the positive control (3.4 dichloroaniline, 4 mg/L). In A1, the embryo presents a malformation on its face, edema and changes in the yolk sac after 24 hours of exposure. In A2 and A3, there are embryos with deformities on the face, edema and yolk sac alteration after 48h and 72h of exposure, respectively. In A4, a larva with edema and changes in the yolk sac is observed after 96 h of exposure.

The images in Figure 1 (B1 – B4) show zebrafish embryos/larvae exposed to the negative control (water from the fish culture system only). In images B1 and B2, the embryos, after 24 and 48 hours of exposure to the negative control, respectively, are healthy and without malformations. In images B3 and B4, the larvae, after 72 and 96 hours of exposure to the negative control, respectively, are also healthy and without malformations.

The images in Figure 1 (C1 – C4) show zebrafish embryos/larvae exposed to 0.05 mg/L of ceftriaxone; in Figure 1(D1 – D4), there are zebrafish embryos/larvae exposed to 0.5 mg/L of ceftriaxone; in Figure 1 (E1 – E4), zebrafish embryos/larvae exposed to 5 mg/L of ceftriaxone; in Figure 1 (F1 – F4), zebrafish embryos/larvae exposed to 50 mg/L of ceftriaxone; and Figure 1 (G1-G4), zebrafish embryos/larvae exposed to 100 mg/L of ceftriaxone.

In C1, D1, E1, F1 and G1, there are zebrafish embryos shown after 24 h of exposure and in C2, D2, E2, F2 and G2, there are zebrafish embryos shown after 48 h of exposure. All of these embryos, exposed from the lowest to the highest tested concentration of ceftriaxone, showed normal development, without malformations.

In C3, D3, E3, F3 and G3, there are zebrafish larvae shown after 72 h of exposure and in C4, D4, E4, F4 and G4, there are zebrafish larvae shown after 96 h of exposure. All of these larvae showed the physiological development of zebrafish, without alterations / malformations during development.

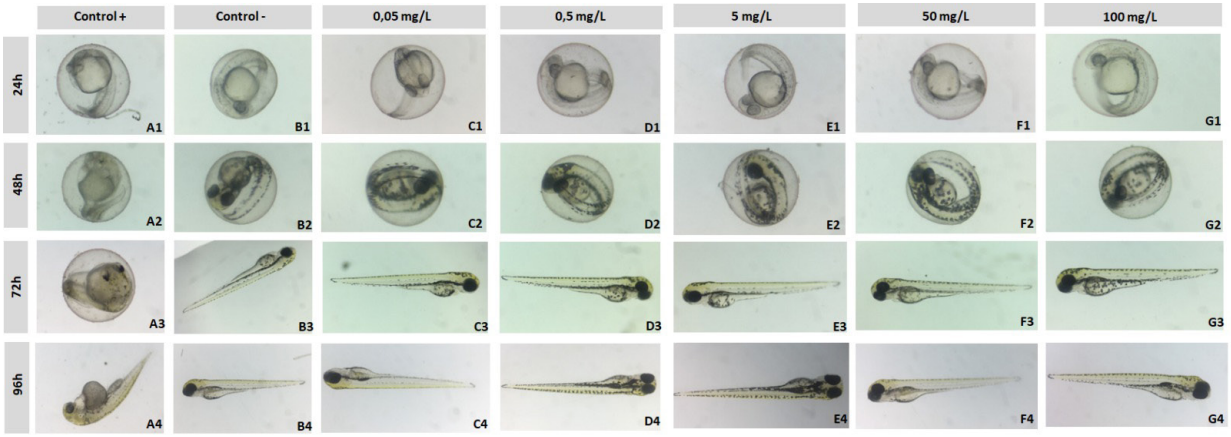

Fig 1. *Danio rerio* embryonic development when exposed to positive control, negative control, and ceftriaxone (0.05; 0.5; 5.0; 50 and 100 mg/L)

Figure 2 shows an overview of the FET. In this image, significant mortality can be observed only in the positive control (C+). Individuals exposed to the negative control and different concentrations of ceftriaxone did not show significant mortality. In this figure, the hatching of fish exposed to different concentrations of ceftriaxone is seen to have occurred on the third day, similarly to the negative control, as happens in the normal development of zebrafish.

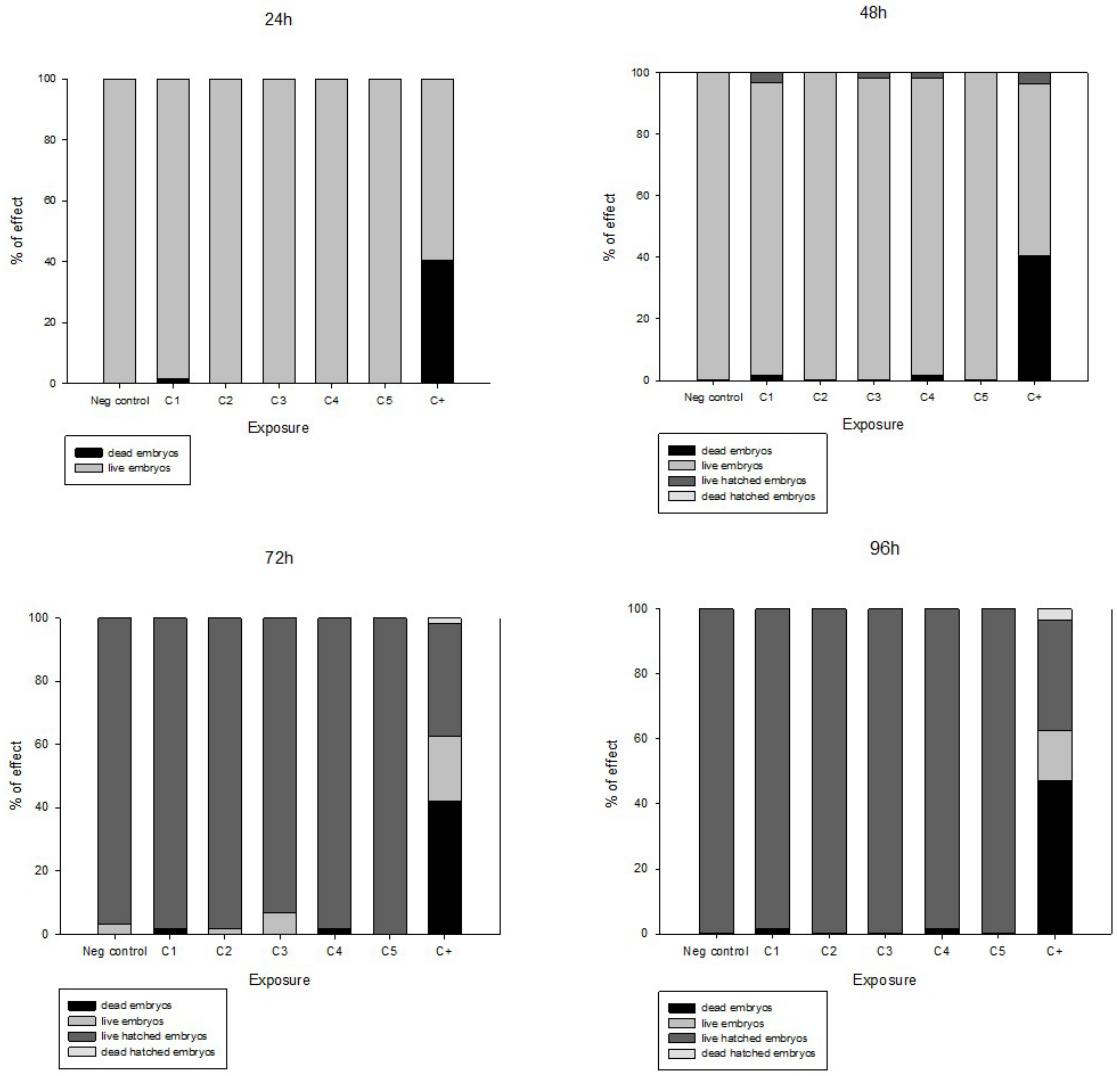

Fig 2. Overview of the effects of ceftriaxone on early life stages of zebrafish over 96 h of exposure

The proportion (in percentage) of dead, alive, and hatched organisms is represented by the different colors of the bars. Neg control = water; C1 = 0.05 mg/L of ceftriaxone; C2 = 0.5 mg/L of ceftriaxone; C3 = 5 mg/L of ceftriaxone; C4 = 50 mg/L of ceftriaxone; C5 = 100 mg/L of ceftriaxone; C+ = positive control (3.4 dichloroaniline, 4 mg/L)

DISCUSSION

The output and subsequent clinical consumption of cephalosporins are vast, and wastewater containing cephalosporin antibiotics leads to a high risk for biological survival in the environment (Das *et al.,* 2019). Therefore, analyzing cephalosporin toxicity in aquatic bioindicators is important.

Zhang *et al.* (2013; 2015) found that cefaclor, cefoperazone, cefotaxime, ceftriaxone (the (Z)-isomer) and cefepime (the (Z)-isomer) showed teratogenicity in zebrafish embryos with ED_{50} values of 1.6 mmol/L (588.4 mg/L), 28 mmol/L (18078.7 mg/L), 30 mmol/L (13664.1 mg/L); 17 mmol/L (9427.8 mg/L), and 18 mmol/L (8650.0 mg/L), respectively. Zhang *et al.* (2015) showed that ceftazidine caused abnormal embryos (e. g. abnormal abdomens, short bodies) at 15 mg/mL (15000 mg/L), and cefotaxime caused abnormal embryos (e. g. yolk invagination and short body length) at 10 mg/mL (10000 mg/L).

The studies carried out by Zhang *et al* (2013; 2015) demonstrated that zebrafish embryo toxicity tests are suitable for analyzing the toxic functional groups of cephalosporins. Besides, these studies demonstrated that cephalosporins, including ceftriaxone, have the potential to cause toxicity in aquatic organisms. However, the concentrations tested by the authors are high, in the order of magnitude of g/L, and even in the worst-case scenario for antibiotic contamination in the aquatic environment, such as antibiotic-producing wastewater, the highest environmental concentrations are in the order of magnitude of mg/L (Larsson, 2014; Yu *et al.*,

2016). According to the literature, the presence of ceftriaxone in the environment was predicted at concentrations of 6.2706 µg/L (Hernández Martínez *et al.,* 2016) and 3.339 mg/L (Magdaleno *et al*., 2014). Thus, the present study analyzed ceftriaxone, a widely used cephalosporin, at concentrations ranging from µg/L to mg/L.

In the present study, no toxicity was observed in zebrafish embryos exposed to concentrations of 0.05 mg/L, 0.5 mg/L, 5.0 mg/L, 50 mg/L and 100 mg/L of ceftriaxone, indicating that this antibiotic is innocuous under the tested conditions.

Although Zhang *et al* (2013) presented an analysis of the teratogenicity of ceftriaxone with a high ED_{50}, the possibility of this antibiotic causing effects at lower concentrations could not be ruled out due to the possibility of a hormesis effect. Hormesis refers to a biphasic dose-response to an environmental agent characterized by a low dose stimulation and a higher dose inhibitory effect (Mattson 2008). However, this effect was not observed at the concentrations tested in this study.

In the present study, the LC_{50} or sublethality/teratogenicity parameters were not calculated, since lethal, sublethal or teratogenic effects were not observed at the concentrations tested. According to OECD guideline 236, a limit test may be performed at 100 mg/L of the chemical under study in order to demonstrate that the LC_{50} is higher than this concentration (OECD, 2013).

In this study, the positive control ensured the correct reading of the test, since, as expected, it showed lethal and sublethal/teratogenic effects. Another important issue was the hatching of embryos because, according to OECD guideline 236, hatching ensures exposure of the embryo without a barrier function of the chorion. Absorption in the embryo occurs through the chorion, but in larvae beyond 3 dpf, absorption occurs through the skin and through swallowing (Zhang *et al.,* 2015). Zhang *et al* (2015) demonstrated the absorption of cephalosporins in early life stages of zebrafish; however, in the present study, no test was carried out to ensure that ceftriaxone was absorbed at the tested concentrations.

Another limitation of the present study is that the medium containing the antibiotic was not changed during the experiment. However, the data in the literature show that ceftriaxone formulated as a solution preparation in sterile water (100 mg/mL) remains stable for three days at room temperature, and remains stable for ten days refrigerated (Owens *et al*., 2003).

Although the present study did not show toxicity of tested concentrations of ceftriaxone in zebrafish early life stages, Dias *et al* (2015) found that cefitriaxone inhibited the growth of *Microcystis aeruginosa*, *Aphanizomenon gracile*, *Chrisosporum berghii* and *Planktotjrix agardhii* at concentrations of 0.2 mg/L, 0.006 mg/L, 0.025 mg/L and 0.1-0.2 mg/L, respectively, which shows the importance of different bioindicators.

Contrary to the initial hypothesis, the results of the present study showed that concentrations of 0.05 mg/L to 100 mg/L of ceftriaxone did not cause toxic effects, as evidenced by the FET test in zebrafish embryos and larvae. However, considering data in the literature in relation to other bioindicators (Dias *et al.,* 2015) and in relation to higher concentrations of ceftriaxone in zebrafish (Zhang *et al.,* 2013), added to the fact that cephalosporin-class antibiotics are widely used, it is still important to be cautious in the use and disposal of ceftriaxone. More studies on the toxicity of cephalosporins at different concentrations using different bioindicators are important for a safer assessment of the effects of these pharmaceuticals on non-target aquatic organisms.

CONCLUSION

The present study, using the FET test, allowed us to conclude that the antibiotic ceftriaxone, in concentrations of 0.05; 0.5; 5.0; 50 and 100 mg/L, did not induce lethal or sublethal effects in zebrafish embryos and larvae.

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AUTHOR CONTRIBUTION

E.M.: Data curation; formal analysis; Investigation; Methodology; Visualization; Writing;

M.F.: Data curation; formal analysis; Investigation; Methodology; Writing; text correction; Supervision;

D.S.: Methodology; Validation; Visualization; Investigation

C.K.G.: Data curation; Investigation; Writing – review and editing.

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