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Comparative study of diclofenac-induced embryotoxicity and teratogenesis in *Xenopus laevis* and *Lithobates catesbeianus*, using the frog embryo teratogenesis assay: *Xenopus* (FETAX)

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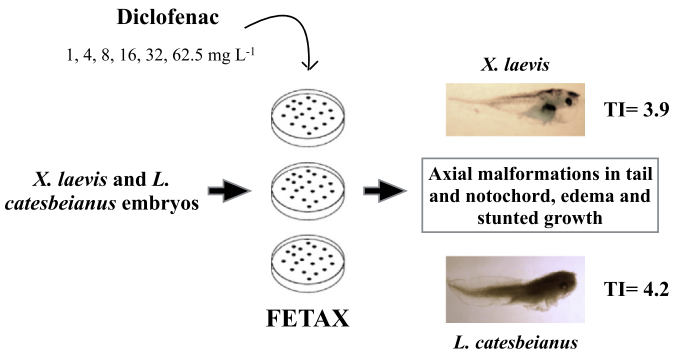
HIGHLIGHTS

- *L. catesbeianus* is marketed as a nutritional meat source in Mexico
- Sensitivity to diclofenac exposure was compared in *X. laevis* and L. *catesbeianus*
- Diclofenac induced embryotoxicity and teratogenesis on L. *catesbeianus* and *X. laevis*
- Axial malformations, edema, and growth inhibition were induced in both species
- *L. catesbeianus* embryos are more sensitive to diclofenac exposure than *X. laevis*

GRAPHICAL ABSTRACT



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ABSTRACT

Water is an increasingly deteriorated, limited natural resource due to population increase and industrialization. Also, the widespread use of pharmaceuticals in modern society leads to their presence in domestic, hospital and industrial effluents. Due to their analgesic properties, some of the most commonly used pharmaceuticals are non-steroidal anti-inflammatory drugs (NSAIDs). High concentrations of one these products, diclofenac (DCF), have been detected in effluents and water bodies of different countries, including Mexico. Diverse studies show that trace amounts (ng L^{-1} to μg L^{-1}) of this compound induce toxicity on aquatic organisms such as algae, microcrustaceans and fish. However, studies on its potential toxicity during development in species of commercial interest such as the American bullfrog *Lithobates catesbeianus* are scarce. The present study aimed to evaluate DCF-induced teratogenesis and embryotoxicity in *Xenopus laevis* and L. *catesbeianus*, a species marketed as a nutritional meat source in Mexico, using the frog embryo teratogenesis assay: *Xenopus* (FETAX). Oocytes in mid-blastula transition were exposed for 96 h to 1, 4, 8, 16, 32 and 62.5 mg DCF L^{-1}. The criteria evaluated were mortality, malformation and growth inhibition. The teratogenic index was 4.2 in L. *catesbeianus*, three-

Abbreviations: DCF, diclofenac; $EC_{50\ (malformation)}$, effective concentration inducing 50% malformation; FETAX, frog embryo teratogenesis assay: *Xenopus*; LC_{50}, median lethal concentration; MBT, mid-blastula transition; NSAID, nonsteroidal anti-inflammatory drugs; ROS, reactive oxygen species; TI, teratogenic index.

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fold higher than the reference limit (1.5), and 3.9 in *X. laevis*. Diclofenac induced diverse malformations in both species, the most frequent of these being axial malformations in the tail and notochord, edema and stunted growth. Results indicate that DCF is a potentially teratogenic compound and is toxic during development in *X. laevis* and L. *catesbeianus*, a species which, due to its sensitivity, can be used to evaluate the toxicity of pharmaceutical products, using FETAX.

1. Introduction

Water is a limited natural resource essential for life and environmental sustainment, which, as a result of rapid social and economic development, has undergone inadequate use and an alarming deterioration (Barceló and De Alda, 2008). In recent years, emerging contaminants (or microcontaminants) have aroused notable interest. These compounds are of diverse origin and chemical nature, and their presence and consequences have until recently gone unnoticed. They are present in water at low concentrations (ng L^{-1} to μg L^{-1}) and are considered harmful to human health and the environment since they can elicit diverse effects in living organisms, such as chronic toxicity, endocrine disruption, and bioaccumulation (VIrkutyte et al., 2010). These compounds have been termed "emerging" because they are still not environmentally regulated or are only now being subjected to regulation (Barceló and De Alda, 2008). A further particularity with regard to this type of contaminants is that, since they are continuously released into the environment due to high production and consumption levels, they need not be persistent to occur environmentally and induce deleterious effects on organisms (Petrović et al., 2003). Among emerging contaminants, those arousing the most concern and study in recent years are pharmaceutical products, nonsteroidal anti-inflammatory drugs (NSAIDs) being the most frequently detected ones in surface water (Buser et al., 1998; Corcoran et al., 2010). Diverse studies state that NSAIDs are a heterogeneous group of medications that are among the most commonly prescribed analgesics, anti-inflammatory agents and antipyretics, and include acetylsalicylic acid, acetaminophen, ibuprofen, diclofenac (DCF) and naproxen. DCF is consumed in the hundreds of tons annually (Buser et al., 1998) and, while non-persistent by reason of its physicochemical properties, due to its continuous release in wastewater discharges it is frequently detected in the environment, inducing diverse potentially negative effects on exposed organisms (Islas-Flores et al., 2013; Oviedo-Gómez et al., 2010; Saucedo-Vence et al., 2015).

NSAIDs are found at higher concentrations in the environment (ng L^{-1} to μg L^{-1}) than other pharmaceuticals (Sim et al., 2010) and environmental analyses of their presence have been carried out in diverse countries and are summarized in various reports (e.g. Richardson, 2007; Palmer et al., 2008; Wang et al., 2011). Their environmental presence is due not only to excreta, through which a significant part of the medication is eliminated from the body without being metabolized, it is also the result of manufacturing processes and the inadequate disposal of residues of these products (Boxall, 2004), not omitting their veterinary, agricultural, livestock and poultry industry use which has continued to grow in recent years (Patiño Menéndez et al., 2014).

Diclofenac is a widely used pharmaceutical in various countries (Petrovic et al., 2008). It is the first-choice anti-inflammatory agent in 74 of 100 countries evaluated by McGettigan and Henry (2013). In Mexico, it is listed in the basic schedule of medications of the public health sector for the treatment of ophthalmological, rheumatological and trauma-related disorders, and has been ranked fourth in total consumption in a family medicine center in the State of Mexico (Gómez-Oliván et al., 2009). This pharmaceutical acts by reversible or irreversible inhibition of one or both isoforms of the enzyme cyclooxygenase (COX-1 and COX-2) which catalyzes the synthesis of diverse prostaglandins (Morrow and Roberts, 2001). These substances are involved in processes such as neurotransmission, ion transport across cell membranes, pain, inflammation, fever, sleep regulation, allergic reactions, muscle contraction, bronchoconstriction, circulatory system regulation and platelet aggregation (Arkhipova et al., 2005; Cha et al., 2006).

Concern about the potential environmental toxicity of DCF emerged some ten years ago with reports linking declining vulture populations in India with veterinary use of this medication (Oaks et al., 2004). Since then, it has been detected around the world at concentrations in the ng L^{-1} to μg L^{-1} range (Lonappan et al., 2016; Nebot et al., 2015; Pereira et al., 2015; Samaras et al., 2013; Tran et al., 2014; Yu et al., 2013). In Mexico only a small number of studies have been carried out in water bodies; those bodies in which DCF has been detected include: the Mezquital Valley irrigation system and the Tula Valley in the state of Hidalgo, with concentrations of 0.25–0.50 μg L^{-1} and 2.052–4.824 μg L^{-1} respectively (Gibson et al., 2010; Siemens et al., 2008); as well as the Lerma-Cutzamala water supply system, with 0.001 μg L^{-1} and 0.028–0.032 μg L^{-1} in ground and surface water respectively (Félix–Cañedo et al., 2013), and Madín Dam with 0.20–031 μg L^{-1} (González-González et al., 2014), both in the State of Mexico. In the latter state, DCF has also been found in industrial effluent from an NSAID manufacturing plant (104.63 μg L^{-1}) and in hospital wastewater (0.0065 μg L^{-1}) (Neri-Cruz et al., 2015; SanJuan-Reyes et al., 2015). As regards DCF-induced toxicity in aquatic organisms, acute toxicity assays show that phytoplankton, with a 96-h median lethal concentration (LC_{50}) of 14.5 mg L^{-1}, is more sensitive to DCF than zooplankton (96-h LC_{50} = 22.43 mg L^{-1}) (Ferrari et al., 2003). In *Oncorhynchus mykiss*, 28 days of exposure to 5 μg DCF L^{-1} induced chronic histopathologic effects such as renal lesions (degeneration of tubular epithelium, interstitial nephritis), while exposure to 1 μg DCF L^{-1} induced gill and subtle subcellular changes such as severe protein accumulation in tubular cells, macrophage infiltration, and structural alterations (dilation, vesiculation) of the endoplasmic reticulum in proximal and distal renal tubules (Schwaiger et al., 2004; Triebskorn et al., 2004). This pharmaceutical has also been shown to induce oxidative stress, and cyto- and genotoxicity in diverse aquatic organisms such as *Hyalella azteca, Daphnia magna* and *Cyprinus carpio* (Gómez-Oliván et al., 2014; Islas-Flores et al., 2013; Oviedo-Gómez et al., 2010). Furthermore, a log K_{ow} of 4.02 to 4.51 has been reported for DCF (Syracuse Science Center, 2002), and its bioaccumulation has been demonstrated in different compartments such as blood plasma, bile, liver, kidney, gills and muscle (Lahti et al., 2011; Mehinto et al., 2010; Kallio et al., 2010; Schwaiger et al., 2004; Saucedo-Vence et al., 2015) and may contribute to the risk of toxicity from exposure to this pharmaceutical in diverse aquatic organisms as it travels through the food chain.

In the past 25 years a dramatic decline in amphibian populations has occurred in many parts of the world, so much so that amphibians are now considered more threatened than mammals or birds (Beebee and Griffiths, 2005). This may be due to their exposure to hazardous levels of contaminants, which are usually present at higher concentrations in irrigation channels, ponds and swamps than in larger water bodies (Tejedo, 2003). As a result, frogs are considered valuable bioindicators, capable of integrating changes in aquatic and terrestrial habitats (Beiswenger, 1988). Ecotoxicological studies reveal that, unlike fish or macroinvertebrates, many amphibian species are particularly sensitive to chemical stress (Birge et al., 2000). Additionally, in the adult stage they feed on invertebrates and are themselves preyed upon by higher