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Evaluation of developmental toxicity and teratogenicity of diclofenac using *Xenopus* embryos

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HIGHLIGHTS

- Diclofenac is a developmental toxicant and teratogen in *Xenopus* embryos.
- Embryos exposed to diclofenac develop various abnormalities.
- Neural tissues are adversely affected by diclofenac.
- Expression of tissue-specific markers is not regulated in RNA transcription level in embryos treated with diclofenac.

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ABSTRACT

Diclofenac is a non-steroidal anti-inflammatory drug (NSAID) with analgesic and anti-pyretic properties. This compound is therefore used to treat pain, inflammatory disorders, and dysmenorrhea. Due to its multimodal mechanism of action and ability to penetrate placenta, diclofenac is known to have undesirable side effects including teratogenicity. However, limited data exist on its teratogenicity, and a detailed investigation regarding harmful effects of this drug during embryogenesis is warranted. Here, we analyzed the developmental toxic effects of diclofenac using *Xenopus* embryos according to the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX) protocol. Diclofenac treatment exerted a teratogenic effect on *Xenopus* embryos with a teratogenic index (TI) value of 2.64 TI; if this value is higher than 1.2, the cut-off value indicative of toxicity. In particular, mortality of embryos treated with diclofenac increased in a concentration-dependent manner and a broad spectrum of malformations such as shortening and kinking of the axis, abdominal bulging, and prominent blister formation, was observed. The shape and length of internal organs also differed compared to the control group embryos and show developmental retardation on histological label. However, the expression of major tissue-specific markers did not change when analyzed by reverse transcription–polymerase chain reaction (RT–PCR). In conclusion, diclofenac treatment can promote teratogenicity that results in morphological anomalies, but not disrupt the developmental tissue arrangement during *Xenopus* embryogenesis.

1. Introduction

Diclofenac [2-(2,6-dichloranilo)phenyl acetic acid] is a phenylacetic acid derivative that is a non-steroidal anti-inflammatory drug (NSAID; Fig. 1A). Like most NSAIDs, diclofenac possesses analgesic, anti-inflammatory, and antipyretic properties. The sodium salt of diclofenac was used to treat osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, and mild to moderate pain in ancient times. Since then, diclofenac has been the most commonly used analgesic in the world and is commercially available in various formulations including ones for oral administration.

Recent studies have shown that diclofenac inhibits the activity of cyclooxygenases and DNA synthesis through multiple mechanisms (Sallmann, 1986; Dastidar et al., 2000; Mastrangelo et al.,

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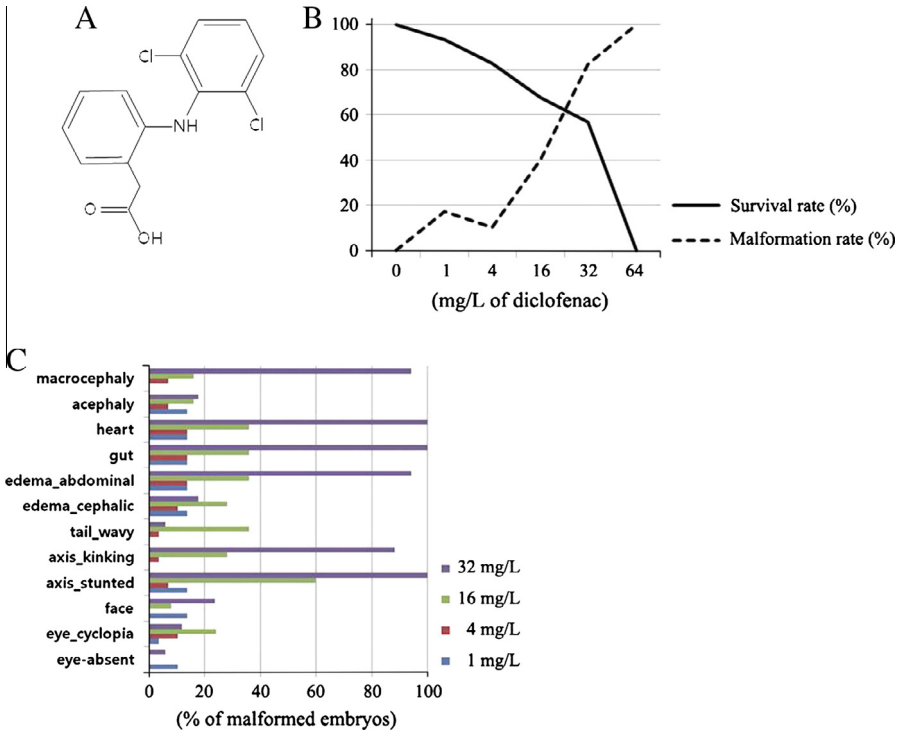

Fig. 1. Dose-dependent effect of diclofenac exposure for 96 h. (A) Lewis structure of the diclofenac used in this study, (B) as the concentration of diclofenac increased, the malformation rate also rose while the survival rate decreased. The MC_{50} was 16 mg L^{-1} and LC_{50} was approximately 32 mg L^{-1} and (C) malformation in various organs was analyzed. Severity (percent with the indicated anomaly) of malformation increased as the diclofenac concentration increased.

2000; Elron-Gross et al., 2008). To regulate immune responses and neuronal function in the brain, diclofenac acts on voltage-gated K^+ channels and acid-sensing ionic channels (Dorofeeva et al., 2008; Villalonga et al., 2010). In rat myoblasts, diclofenac prevents the influx of Na^+ via the inhibition of voltage-gated Na^+ channels (Fei et al., 2006), while promoting Ca^{2+} efflux from mitochondria (Li et al., 2009). These studies suggest that the multimodal mechanisms of diclofenac action make it potential compound that exerts ~~a~~ wide range of physiologic effects and known most commonly prescribed analgesic in the world. Conversely, the properties of diclofenac may require reevaluation to ensure patient safety although this drug is generally considered safe for human use at ~~the~~ recommended doses.

Diclofenac has been proven as a pregnancy risk class C drug by the United States Food and Drug Administration (FDA). Even though the toxicity and teratogenicity of diclofenac were measured, conflicting data have been published for different animal model systems. Fetal neuronal cells apoptosis is significantly induced in diclofenac-treated pregnant rats (Gokcimen et al., 2007). Additionally, diclofenac-treated rodents deliver fetuses with severe morphological abnormalities such as defects of the palate, limbs, and ductus arteriosus (Montenegro and Palomino, 1990; Zenker et al., 1998; Rein et al., 1999; Chan et al., 2002). Diclofenac-treated medaka fish embryos also have decreased survival rates, shrunken yolks, and hemorrhage (Nassef et al., 2010). Furthermore, myofibril misalignment occurs in diclofenac-treated zebrafish embryos via disruption of actin organization (Chen et al., 2011) and alteration of mRNA expression (Felice et al., 2012). Deregulation of mitochondrial functions and abnormal apoptosis-related gene expression have been observed through differential mRNA and transcriptome analyses (Felice et al., 2012). These studies clearly suggest diclofenac toxicity during embryogenesis, although it seems to be safe for embryos at considerably low doses. As this compound is frequently prescribed to treat dysmenorrhea or menorrhagia in young women, adequate and well-controlled studies should be conducted to evaluate potential teratogenic effects. Developmental toxicity and teratogenecity associated with diclofenac might or would have dramatic impacts on embryogenesis and detailed mechanisms of underlying these effects should be assessed.

Here, we present data from molecular and pathological assays, which indicates that diclofenac is a developmental toxicant and teratogen. Diclofenac administration causes neural tissue underdeveloped in terms of size and shape as compared to normal developmental process. We determined that diclofenac is a developmental toxicant and teratogen in *Xenopus* embryos using a Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX) assay. In addition, diclofenac-treated embryos had various developmental abnormalities and defects of various organs including neural tissues.

2. Materials and methods

2.1. Chemicals and FETAX solution

All analytic-grade reagents, diclofenac sodium salt ($C_{14}H_{10}Cl_2NNaO_2$), human chorionic gonadotropin (HCG), and 3-aminobenzoic acid ethyl ester (MS222) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The FETAX control solution contained 10.7 mM NaCl, 1.14 mM $NaHCO_3$, 0.4 mM KCl, 0.1 mM $CaCl_2$, 0.35 mM $CaSO_4{\cdot}2H_2O$, and 0.3 mM $MgSO_4$ in deionized water; the pH was adjusted to 7.6–7.9. A diclofenac stock (10 mg mL^{-1}) was freshly made and diluted in FETAX solution.

2.2. Animals

Adult *Xenopus* were purchased from Nasco (Nasco, Fort Atkinson, WI, USA) and housed in an aquarium with triple-filtered tap water at 18 ± 2 °C and an alternating 12-h light/dark cycle. The animals were fed a semi-synthetic diet (Nasco, Fort Atkinson, WI, USA).