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Developmental Toxicity of Diclofenac and Elucidation of Gene Regulation in zebrafish (Danio rerio)

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Environmental pollution by emerging contaminants, e.g. pharmaceuticals, has become a matter of widespread concern in recent years. We investigated the membrane transport of diclofenac and its toxic effects on gene expression and the development of zebrafish embryos. The association of diclofenac with the embryos conformed to the general partition model at low concentration, the partition coefficient being 0.0033 ml per embryo. At high concentration, the interaction fitted the Freundlich model. Most of the diclofenac remained in the extracellular aqueous solution with less than 5% interacting with the embryo, about half of which was adsorbed on the membranes while the rest entered the cytoplasm. Concentrations of diclofenac over 10.13 μM were lethal to all the embryos, while 3.78 μM diclofenac was teratogenic. The development abnormalities at 4 day post treatment (dpt) include shorter body length, smaller eye, pericardial and body edema, lack of liver, intestine and circulation, muscle degeneration, and abnormal pigmentation. The portion of the diclofenac transferred into the embryo altered the expression of certain genes, e.g. down-regulation of *Wnt3a* and *Gata4* and up-regulation of *Wnt8a*. The alteration of expression of such genes or the regulation of downstream genes could cause defects in the cardiovascular and nervous systems.

The occurrence of pharmaceuticals in the environment has become a matter of widespread concern in recent years because of their continuous discharge and potential subtle toxicity[1]. Diclofenac (sodium 2-[2-(2,6-dichloroanilino)phenyl]acetate) is one of the most commonly used non-steroidal anti-inflammatory drugs (NSAIDs), acting as an inhibitor of the cyclooxygenase responsible for the synthesis of prostanoids[2]. Diclofenac is widely used to relieve pain and inflammation caused by arthritis, gout, dysmenstruation, menorrhagia, and other conditions[3]. The global consumption of diclofenac is estimated at 940 tonnes per year in the form of capsules, suppositories, tablets, intravenous solution and ointments[4,5]. Some diclofenac is not completely metabolized after consumption and is discharged to wastewater treatment plants, and subsequently to the receiving water body because it is not completely removed by the plants[4]. Diclofenac has been detected in municipal wastewater effluent, surface water, groundwater, and drinking water at concentrations in the order of ng/L to μg/L (10^{-6} to 10^{-3} μM)[6–9]. Although diclofenac at these environmental concentrations could not cause lethal effects on organisms, chronic toxicity is potentially possible. After 28 days' exposure to 3.38×10^{-3} μM diclofenac, cytological alterations were observed in the liver, kidney and gills of rainbow trout (*Oncorhynchus mykiss*); the lowest observed effective concentration (LOEC) for renal lesions was 1.69×10^{-2} μM[10,11]. Cytological alterations also occurred after 21 days in brown trout (*Salmo trutta f. fario*) exposed to 1.69×10^{-3} μM diclofenac[12]. The dramatic decline of the number of vultures in India and Pakistan since the 1990s was also reported to be related to their exposure to diclofenac residues. Diclofenac was widely used on cattle and water buffalo in these areas to reduce their inflammation caused by trauma and infectious disease[13]. After vultures have scavenged diclofenac-treated livestock cadavers, the drug accumulates in their bodies, causing renal failure and death[4,14]. Hence, diclofenac could pose a potential risk to ecosystems. Further study of its toxicity is imperative.

The zebrafish (*Danio rerio*) is a small, freshwater tropical fish which is easy to grow and maintain, and has short growth period and high fecundity. The zebrafish model is more readily manipulated in large-scale genetic or chemical screens and less subject to legal and ethical restrictions than mammalian models[15]. Moreover, most zebrafish genes have orthologs in the human, and the high conservation of key developmental genes indicates common molecular pathways during development[16,17]. Therefore, the zebrafish has long been used as a test model

to evaluate the environmental and human health risk of chemicals. The zebrafish early life stage (ELS) test is one of the most popular tools for evaluating the acute or chronic toxic effects of aquatic pollutants on fish. It considers reactions of the most sensitive stage in fish development, providing a wide variety of developmental parameters as the endpoints of toxicant effects including development delay, development of somites, eyes and otoliths, spontaneous movement, circulation, heart rate, pericardial edema, yolk sac edema, hatching, and the length and deformation of the tail[18,19]. Toxicogenomic studies using the zebrafish have also been used recently to elucidate the toxic effects of pollutants on fish development at the molecular level[20–22].

The interaction between pollutants and endogenous molecules is the internal cause of toxicity. However, the pollutants must first penetrate the cell membrane before they interact with intracellular target molecules to induce the subsequent occurrence of toxicity. Thus, transmembrane transports of pollutants is the fundamental step in their toxicological effects[23]. In this study, zebrafish embyos were exposed to diclofenac. The aim was to elucidate the transmembrane transport process of diclofenac and its subsequent developmental and genetic toxicity.

Results

Association of diclofenac with embryos. Twenty embryos were exposed to diclofenac (0.3–1620 μM) to investigate the interaction between diclofenac and embryos. As illustrated in Fig. 1A, the association (γ) of diclofenac with the embryo increased steadily with increase of initial concentration (C_0), while the trend was slower at high initial concentration. In order to elucidate the association characteristic of diclofenac, thermodynamic models were applied to fit the association process at various concentrations. Association (γ) increased linearly with C_0 between 0.3 and 162 μM diclofenac. The general partition model was used to describe this association (Fig. 1B) ($R^2 > 0.99$). The partition coefficient (P) was calculated as 3.3 μl per embryo. When C_0 increased from 162 to 1620 μM, the association fitted the Freundlich model (Fig. 1C). From the plot of log γ *vs.* logC_f ($R^2 > 0.98$), the heterogeneity factor ($1/n$) was calculated to be 0.6972.

The effects of pH, ionic strength, and temperature on the association of diclofenac with the embryo were investigated. The amount of diclofenac bound to the embryo increased and approached a maximum as the ionic strength increased from 0 to 0.15 M (Fig. S3). The amount associated with the embryo decreased when NaCl exceeded 0.15 M. Increased ionic strength often favors hydrophobic interactions, thus facilitating the association of diclofenac with the embryo, while a higher concentration of electrolyte weakens embryo activity and thus affects the binding of diclofenac[28]. Higher temperatures favored the association of diclofenac with the embryo because an increase of the membrane flow rate accelerated metabolic activity[28]. However, more than 40°C could affect embryo activity adversely, so the amount of diclofenac associated was slightly reduced. The amount of bound diclofenac fluctuated with increasing pH with no trend.

The distribution of diclofenac (5–162 μM) among the different parts of the embryo was determined by fragmenting the embryo and measuring the diclofenac concentrations. Most of the drug remained in the extracellular aqueous solution; less than 5% was associated with the embryo (Fig. S4). Of the diclofenac associated with the embryo, about 53% was adsorbed on the membranes while rest entered the cytoplasm (Fig. 2A). With increasing exposure concentration, the amounts of diclofenac on the membrane and in the cytoplasm increased almost linearly, with $P = 0.0016$ and 0.00145 μl per embryo, respectively (Fig. 2B).

Effect of diclofenac on the development of zebrafish embryos. The mortality of embryos at different exposure times (0–4 d) was recorded. Only one embryo died at 1 dpt in the 1.01 μM diclofenac treatment (Table S3). In the 3.38 μM treatment, the embryo lethality was about 26.7% at 4 dpt. However, the embryos began to die at 3 dpt when exposed to 10.13 and 15.20 μM diclofenac, and both treatments were 100% lethal at 4 dpt.

No morphological abnormality was observed in the embryos at 1 dpt in the 1.01 and 3.38 μM treatments. However, severe tail malformation occurred in the 10.13 and 15.20 μM treatment groups (Fig. 3), with slight pericardial edema. At 2 dpt, the zebrafish embryo had a slight pericardial edema when exposed to 3.38 μM diclofenac, while severe tail malformation and pericardial edema were observed in the 10.13 and 15.20 μM groups (Fig. 3). After 3 d exposure, severe pericardial edema and muscle degeneration were observed in the 3.38 μM treatment group. Embryo deaths occurred in the 10.13

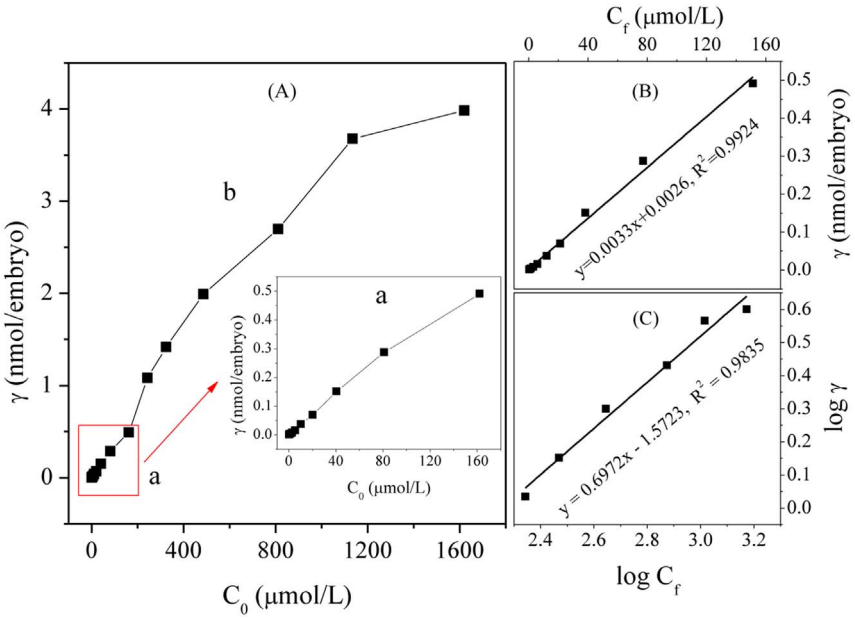

Figure 1 | Binding number of diclofenac to the zebrafish embryo with C_0 increasing from 0.3–1620 μM. (A) a, partitioning stage; b, Freundlich adsorption stage; (B) Plot of γ vs C_f (C_0 from 0 to 162 μM); (C) Plot of *log* γ vs *log* C_f (C_0 from 162 to 1620 μM).

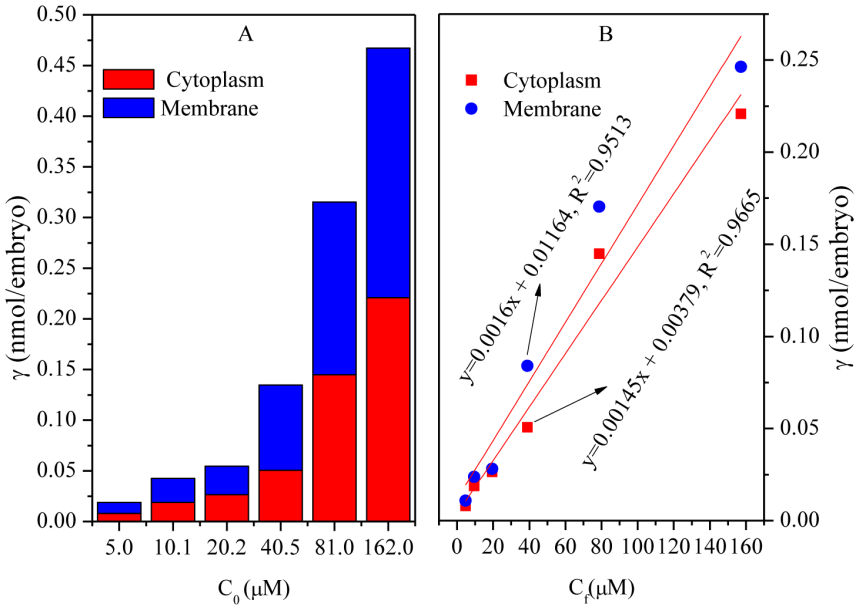

Figure 2 | Distribution of diclofenac in the cytoplasm and membrane of zebrafish embryos after 8 h exposure. The concentration of diclofenac was between 5 and 162 μM. (A) plots of γ vs C_0; (B) plots of γ vs C_f.

and 15.20 μM treatment groups, with mortality rates of 13.3 and 43.3%, respectively; while the survivors had severe trunk curvature, pericardial edema, and muscle degeneration (Fig. 3). At 4 dpt, all the zebrafish in the 10.13 and 15.20 μM groups died, while normal development was observed in all zebrafish in the 1.01 μM treatment group (Table S4). In the 3.38 μM treatment group, the toxicity-related characteristics included: shorter body length (Fig. 3), smaller eye, muscle degeneration, lack of liver, intestine and circulation (Fig. S5), pericardial and body edema (Fig. S6), and abnormal pigmentation (Fig. S7).

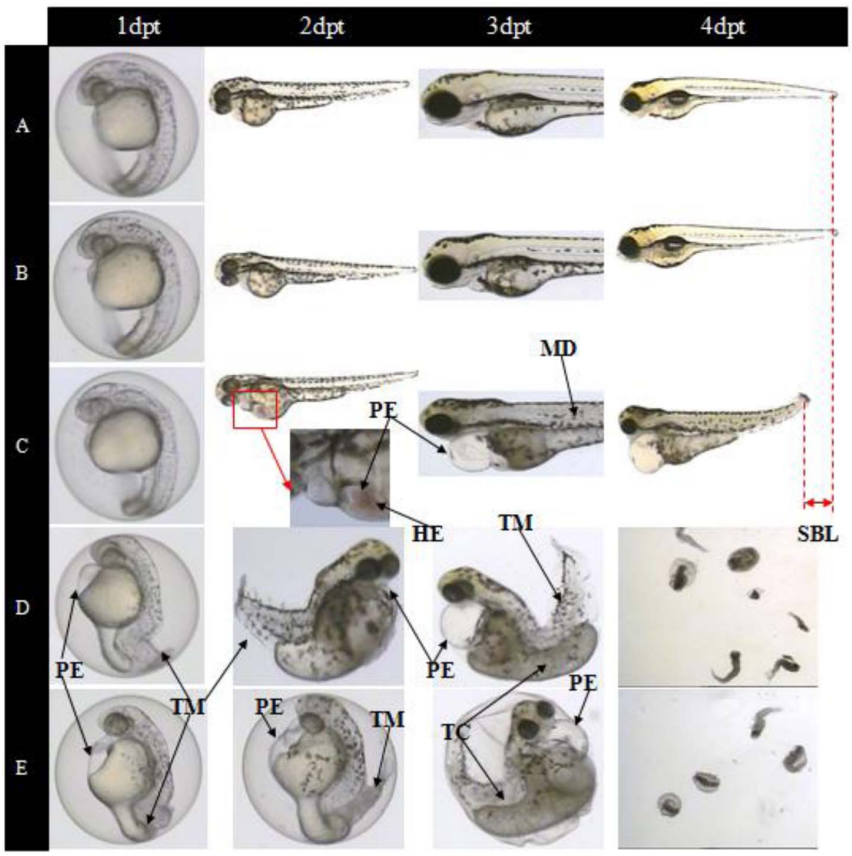

Figure 3 | Toxic effect of diclofenac on zebrafish during the exposure at 1–4 dpt. (A) Control group; (B) 1.01 μM exposure group; (C) 3.38 μM exposure group; (D) 10.13 μM exposure group; (E) 15.2 μM exposure group. HE: hemagglutination, MD: muscle degeneration, PE: pericardial edema, SBL: short body length, TC: trunk curvature, TM: tail malformation.

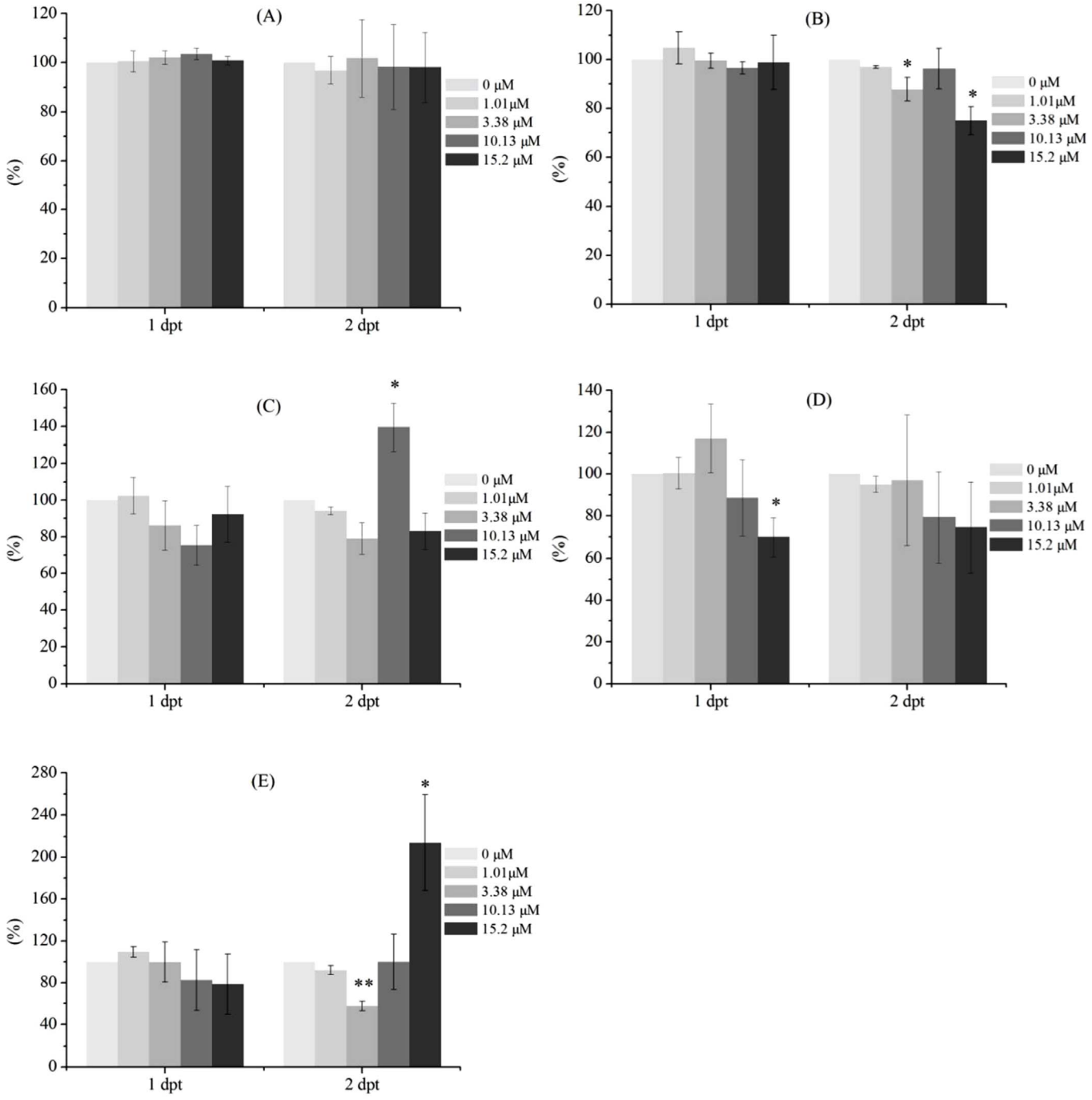

Figure 4 | Expression profiles of β-actin (A), *Wnt3a* (B), *Wnt8a* (C) *Gata4* (D) and *Nkx2.5* (E).

Effect of diclofenac on gene expression. As the reference gene, β-actin expression was stable (Fig. 4A) and the band was very bright, with a consistent relative optical density (Fig. S8a). Thus, normalization of the experimental samples in the experiment was consistent, confirming the reliability of the subsequent target gene expression analysis. No band was observed on the blank control lane in the gels (Fig. S8B), thus the contaminant RNA interfering was negligible when the cycle number in the PCR was greater than 30. Diclofenac had an insignificant inhibitory effect on *Wnt3a* and *Wnt8a* expression at 1 dpt. However, at 2 dpt, the expression of *Wnt3a* in the 3.38 and 15.20 μM treatments was significantly less than in the control (Fig. 4B, AGE was shown in Fig. S8b), while the expression of *Wnt8a* in the 10.13 μM treatment was notably greater (Fig. 4C, AGE was shown in Fig. S8c). At the higher diclofenac concentrations, *Gata4* expression showed a trend towards inhibition, but this was significant only in the 15.20 μM treatment at 1 dpt (Fig. 4D, AGE was shown in Fig. S8d). The expression of *Nkx2.5* was stable at 1 dpt, but was inhibited at 2 dpt in the 3.38 μM treatment and increased at higher concentrations (Fig. 4E, AGE was shown in Fig. S8e).

Discussion

The membrane is an essential component of the cell, consisting mainly of a phospholipid bilayer, membrane proteins, and oligosaccharides. It is the natural barrier preventing the free entry of external molecules into the cell, thus maintaining the stability of the internal environment. Furthermore, the cell membrane has other essential functions such as signal transduction, nutrient transport and so on.

Exogenous pollutants interact first with the cell membrane before they exert detrimental effect on the organism, so the study of transmembrane transport helps to elucidate the mechanism of toxicity. Owing to the presence of benzene and chloride residues in the molecule, diclofenac could theorically distribute into the fatty acid chain region of the embryo membranes by hydrophobic interaction at lower concentrations (0.3–162) μM. Under physiological conditions, diclofenac is mostly negatively charged (pK_a = 4.15). The negatively charged molecule could also interact with the $-NR_4^+$ groups of the membrane bilayer by electrostatic interaction. Furthermore, diclofenac might interact with polar groups such as -COOH and $-NH_2$, on the embryo membrane via hydrogen bonds and van der Waals forces. At higher concentrations, between 162 and 1620 μM, diclofenac could not only partition into the membrane layer but also adsorb on the membrane surface by joint non-covalent interactions. Results indicated that, like other hydrophobic drugs in transmembrane transport studies, e.g. chloramphenicol, most of the drug remained in the extracellular solution[28]. However, only half of the diclofenac associated the membrane was transported into the cytoplasm, compared to over 80% of the adsorbed chloramphenicol[28]. The different distribution behaviors of these drugs might be related to membrane lipid composition and the lipid-water partition coefficients. The lipid-water partition coefficients for membrane phospholipids are reported to be lower than those for lipids stored in the cytoplasm[28,29], so diclofenac might be more difficult to distribute into the storage lipids of the yolk in the cytoplasm than chloramphenicol because the sodium salt of diclofenac was used in this study. Although little diclofenac was transported into the embryo, this could be nevertheless detrimental to normal development.

In the ELS test study, the 4 d LOEC for diclofenac was between 1.01 and 3.38 μM, lower than previous reports of 10 d LOEC 25.15 μM[30] and 3 d LOEC 4.71 μM[31]. Hallare et al. reported a tendency to delay in the 3.14 and 6.29 μM exposure groups after 96 h, with no significant mortality or malformation during the exposure period[32]. Delayed hatching was also observed up to the end of 144 h exposure[5], while the late-hatched embryos had different morphologies from the normally hatched ones, such as hydro-edema. Hatching and growth retardation, as well as yolk sac and tail deformation, were recorded in another study with concentrations of diclofenac above 4.71 μM[31]. In addition to edema, tail deformation, other sublethal effects were newly observed, including shorter body length, smaller eye, lack of liver, intestine, and circulation, muscle degeneration, and abnormal pigmentation. The concentration of diclofenac in this study was one million times higher than the environmental concentration, thus the acute toxicity risk of diclofenac in the environment is quite low. Although the concentration of diclofenac in the environment is low, which is ordinarily in the order of 10^{-6} μM, there is a steady input of the drug into the environment, considered as a pseudo-persistent compound, so organisms are continuously exposed. Furthermore, thousands of chemicals co-exist in nature, and organisms are exposed to a combination of stressors. Hence a negative effect of chronic exposure in the environment remains possible.

The *Wnt* genes encode secreted glycoproteins that play a significant role in body patterning, cell proliferation/differentiation and tumorigenesis by acting at the cell surface or on the extracellular matrix to mediate cell-cell signaling[24,33]. Both *Wnt3a* and *Wnt8a* function in body patterning during early embryogenesis. Inhibition of *Wnt8a* in zebrafish resulted in posterior body reduction and expansion of dorsal axial tissues, indicating that it is involved in the formation of the paraxial mesoderm and tail[24,34,35]. Loss of *Wnt8a* function has also been reported to prevent tail development[36]. However, the injection of *Wnt8a* RNA led to dose-dependent changes in *gsc* and *ntl* expression, causing defects involving mesoderm and axis formation. Thus, *Wnt8a* is presumed to be a factor involved in specifying the expression of *gsc* and other genes, inducing the establishment of the embryonic axis[33]. Deficiency of *Wnt3a* leads to a reduction of the somite and posterior body structures in mouse embryos[24,37]. Our present results demonstrated that treatment with diclofenac inhibits *Wnt3a* expression but up-regulates *Wnt8a* expression. *Wnt3a* and *Wnt8a* regulate a number of downstream genes, such as ventrally expressed homeobox genes and cdx genes to mediate the *Wnt* signals. Because *Wnt* signaling plays an important role in body patterning during early vertebrate embryogenesis, the up- or down-regulations of *Wnt* genes could induce the abnormal body patterning of zebrafish embryo, thus trunk curvature and tail malformation were observed after exposure to diclofenac in this study.

The homeodomain transcription factor *Nkx2.5* and the zinc-finger transcription factor *Gata4* are two of the earliest markers of precardiac cells. They play critical roles in the induction of the cardiac program[27,38]. *Nkx2.5* was reported to be associated with the determination of myocardial cell fate and the initiation of the cardiogenic differentiation program[38,39]. Loss of *Nkx2.5* in mice led to impaired cardiac looping and defective gene expression related to cardiac muscle, while lack of *Gata4* caused the disruption of late cardiac morphogenetic movements[38,40,41]. Alterations in *Nkx2.5* and *Gata4* expression result in defects of cardiac differentiation and the heart[42–44]. In the present study, the expression of *Gata4* was down-regulated at 1 dpt, especially in the 15.20 μM treatment. Although *Nkx2.5* was expressed stably at 1 dpt, it was inhibited at 2 dpt after exposure to 3.38 μM. The down-regulation of *Gata4* and *Nkx2.5* might lead to defective gene expression related to cardiac muscle, and affect the cardiogenic differentiation program, thus the severe pericardial and body edema was observed after exposure to diclofenac. Interestingly, the expression of *Nkx2.5* was significantly up-regulated with increasing diclofenac concentration, which might be ascribed to the requirement of *Nkx2.5* for repair of the heart after impairment. Therefore, *Nkx2.5* and *Gata4* are presumed to be candidate genes for heart defects following diclofenac treatment. The alteration of *Nkx2.5* and *Gata4* expression might be the molecular basis of zebrafish embryo toxicity after diclofenac exposure.

Overall, the transmembrane transport of diclofenac and its subsequent developmental and genetic toxicity were illustrated in Fig. 5. Diclofenac was predominantly distributed in the extracellular solution with little partitioning into the phospholipid bilayer. After entering the phospholipid layer, it could be further partitioned into the cytoplasm owing to the presence of storage lipids in the yolk. After transfer into the developing cells, it would interact with macromolecules, e.g. DNA, inducing the alteration of expression of genes such as *Wnt3a*, *Wnt8a*, *Gata4*, and *Nkx2.5*. The expression of such genes was important to the development of the cardiovascular and nervous system. Thus, the altered expression of such genes or the regulation of downstream genes could cause defects in the cardiovascular and nervous systems, such as pericardial and body edema, shorter body length, trunk curvature and tail malformation and so on. The zebrafish genome has important similarities to the human genome, and the developmental process and physiological functions are conserved and similar to those in the human. The zebrafish is a great model to investigate human health risk. As zebrafish embryo development is sensitive to diclofenac, diclofenac in the environment could also have potential risks for the development of human embryos, which could indicate toxicological effects on human health.

Methods

Experimental design. In order to investigate the association of diclofenac with membranes, different concentrations of diclofenac were prepared by diluting with reconstituted water and then incubated with the embryos. After the fragmentation of embryos, the concentrations of diclofenac in the extracellular solution, membrane and cytoplasm were determined by HPLC-UV or UPLC-MS/MS, so the transmembrane distribution of diclofenac in different parts of the embryo could be evaluated. According to the developmental toxicity experiment, the target organs of developmental toxicity were mainly the cardiovascular and nervous systems. It could be speculated that the molecular basis of toxicity was related to the effect of diclofenac

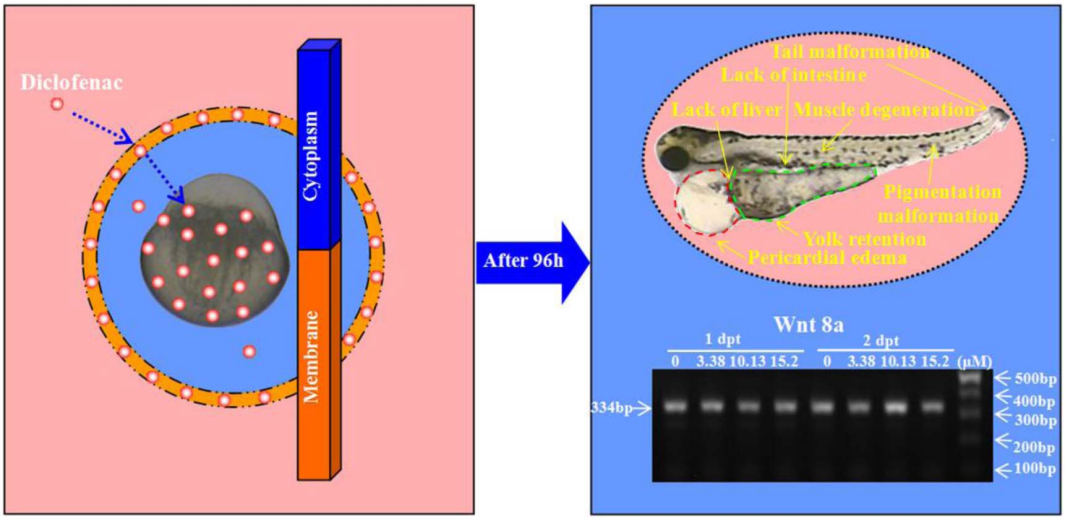

Figure 5 | Illustration of transmembrane transport of diclofenac and its subsequent developmental and genetic toxicity. (The gels and blots of *Wnt8a* were cropped and the full-length blots/gels were presented in Supplementary Figure S9).

on the expression of *Wnt* pathway genes and the development of the cardiovascular system. The gene of *Wnt3a* (wingless-related MMTV integration site 3A) was reported to be related with development of heart and body axis[24,25], while *Wnt8a* (wingless-type MMTV integration site family, member 8a) was related to the development of body axis and tail[24,26]. Expression of *Gata4* (*Gata* binding protein 4), and *Nkx2.5* (Nk2 homeobox 5) were associated with the induction of the cardiac program, and important to the development of the cardiovascular system[25,27]. We therefore conducted PCR to investigate the expression levels of selected genes: *Wnt3a, Wnt8a, Gata4*, and *Nkx2.5*. Because the embryos began to die in the 10.13 and 15.20 μM treatments at 3 dpt and all the embryos died at 4 dpt, the gene expression changes were observed at 1 and 2 dpt.

Apparatus and materials. High performance liquid chromatography (HPLC) (Agilent HPLC 1200, Agilent, USA) with a UV detector was used to determine the concentration of diclofenac. Ultra performance liquid chromatography (UPLC) (Thermo Accela UPLC, Thermo Fisher Scientific, USA) coupled with a triple quadrupole mass spectrometer (TSQ Quantum Access, Thermo Fisher Scientific, USA) was used to measure low concentrations of diclofenac in membranes and cytoplasm. An ultrasonic cell disruptor (Model JY92-II, Ningbo Scientz Biotechnology Company, China) was used to disrupt the embryos. A centrifuge (TGL-16M, Changsha Xiangyi Centrifuge Instrument Company, China) was used to separate diclofenac from suspensions after ultrasonication. A dissecting microscope (SMZ645, Nikon, Japan) fitted with a digital camera (JVC, Nikon, Japan) was used to observe the toxicity-related changes in the embryos following exposure. The horizontal electrophoresis apparatus (DYY-6C, Beijing Liuyi Instrument, China) with gel documentation and analysis system (JS680, Shanghai Peiqing Science & Technology, China) was used for agarose gel electrophoresis (AGE). A PCR system (TC-96/G/H(b)A, Bioer, Japan) was used for PCR analysis. All the ultra pure water was produced by Millipore Milli-Q Ultrapure Gradient A10 purification system.

A 3.38 mM solution of diclofenac (diclofenac sodium salt, D6899, Sigma-Aldrich, USA) was prepared in ultra pure water and was diluted to the desired concentrations before use. Reconstituted water was prepared by dissolving $CaCl_2.2H_2O$ (0.294 g), $MgSO_4.7H_2O$ (0.123 g), $NaHCO_3$ (0.065 g), and KCl (0.006 g) in ultra pure water (1 L). Phosphate buffers (0.1 M) at pH 5.5, 6.5, 7.5, 8.5, 9.5 were prepared to study the effect of pH on the transmembrane transport of diclofenac. NaCl solutions from 0 to 0.25 M were prepared to evaluate the effect of ionic strength.

All of the experimental protocols were carried out following the approved protocol by Animal Care and Use Committee of Tongji University. Wildtype zebrafish with genotype AB were kept in a 25 L rectangular tempered glass tank containing water with the following characteristics: pH 7.5 ± 0.5, 250 mg/L $CaCO_3$ hardness, 10.5 ± 0.5 mg/L dissolved oxygen, at 26 ± 0.5°C. The photoperiod was adjusted to a 14-h light/10-h dark cycle. Fish were fed with commercially available frozen red mosquito larvae twice daily. On the evening before each test, male and female adult zebrafish (2 : 1) were placed in several spawning boxes each containing a mesh laid at the bottom of the tank. Spawning was triggered once the light was turned on and was completed within 30 min. The fertilized eggs at the bottom of the spawning boxes were collected with a glass siphon and washed three times with oxygen-saturated reconstituted water. Unfertilized or irregular eggs were separated from the normally-developing ones. Fertilized embryos were transferred to the test solution not later than two hours post fertilization.

Transmembrane transport of diclofenac. Twenty embryos were exposed to 2 ml diclofenac at each concentration. After 8 h, the supernatant was sampled to determine the concentration of diclofenac (C_f) by HPLC-UV (Test S1). The treated embryos were separated, and rinsed with water, then suspended in 2 ml ultra pure water and ultrasonicated for 90 s at 0°C with probe-type sonicator at 120 W, with intervals of 10 s sonication and 5 s rest. The sonicated mixture was centrifuged at 6000 rpm for 5 min, and the supernatant was separated to determine the amount of diclofenac distributed in the cytoplasm (C_c) by UPLC-MS/MS due to its lower detection limit (Test S2). The membrane pellets were re-suspended in 2 ml methanol and sonicated at 240 W for 90 s, with six cycles of 10 s sonication and 5 s rest. The mixture was further centrifuged at 12000 rpm for 5 min, and the supernatant was sampled to determine the concentration of diclofenac by UPLC-MS/MS; which was considered as the membrane-associated diclofenac (C_m). The blank control was conducted in the reconstituted water throughout all the experimental procedures as described above. To evaluate the recovery of diclofenac in different parts of embryo during the extraction, a known amount of diclofenac was added in the blank control at each step. The recovery of diclofenac was calculated to be 96.2, 94.2, and 93.6% in the extracellular solution, membrane and cytoplasm, respectively.

Identification of development toxicity. Thirty zebrafish embryos were used per treatment. They were distributed in 6-well microplates (Nest Biotech, China), which were placed in an incubator with the temperature controlled at 26 ± 0.5°C and a 14-h light/10-h dark photoperiod. Five treatments were used: control, and diclofenac at the nominal concentrations 1.01, 3.38, 10.13, and 15.20 μM. The embryos were observed under the dissecting microscope at 1, 2, 3 and 4 days post treatment (dpt) and photographed with the digital camera. Dead zebrafish were recorded and promptly removed from the solution during observations. The following parameters were evaluated: pericardial and body edema, abnormal pigmentation, size of eye, body length, heart-, head-, tail-, otoliths- and muscle deformation, absence of liver and intestine, and bleeding. After the treatments, all the zebrafish at different development stages were killed with 0.25 g/L tricainemethanesulfonate, which conformed to the American Veterinary Medical Association (AVMA) requirements for killing by anesthetic. The data were analyzed by Fisher's exact test, with a threshold significance level of $p < 0.05$.

Analysis of mRNA expression by quantitative polymerase chain reaction (qPCR). Two hundred embryos were exposed to each treatment of 0, 1.01, 3.38, 10.13 and 15.20 μM diclofenac until 2 dpt. At 1 and 2 dpt, 100 embryos were randomly selected from each treatment and total RNA was extracted with TRIzol reagent (Sigma-Aldrich, USA) after rupture of the membranes. Then the RNA extract was analyzed by agarose gel electrophoresis (AGE), and the bands of 5S, 18S, and 28S were clearly visible (Fig. S2). The total RNA concentrations were determined by spectrophotometry (SP-752, Shanghai Spectrum Instruments Company, China) at 260 nm. After dilution by a factor of 300, the A 260/280 ratio of the RNA solution was between 1.62 and 1.72 (Table S1). The RNA solution was diluted to 1 μg/μl, and 10 μl diluted RNA sample was taken and reverse-transcribed into double-strand cDNA with a ReverTra Ace-α-reverse transcription kit (Toyobo, Japan). cDNA was amplified with 2 × PCR MasterMix (BioTeKe Corporation, China) on the PCR system. The process of PCR was as follows:

20 μl reaction mixture:		Reaction process (34 cycles):	
PCR MIX	10 μl	95°C	2 min
Primer forward	0.5 μl	94°C	30 s
Primer reverse	0.5 μl	45–60°C	30 s
cDNA	0.8 μl (0.8 μg)	72°C	30 s
Ultra pure water	8.2 μl	72°C	10 min
		4°C	∞

The temperature yielding an obvious and pure band was selected as the optimum annealing temperature, as shown in Table S2. According to the optimum annealing temperature, the reaction cycles of β-actin (reference gene) were set at 20–34, while those for *Gata4* and *Nkx2.5* were set at 26–40 and *Wnt3a* and *Wnt8a* at 28–42. A sample was taken every other cycle with eight samples in total. In each case the reaction was followed by AGE and photography. According to the optical density of the target band, the cycles yielding high optical density before the plateau stage were confirmed as the optimum cycles, as displayed in Table S2.

According to the optimum annealing temperature and cycles, PCR was conducted in triplicate for each gene in every sample, followed by AGE and photography. The optical density of each was measured using the software. The relative optical density was taken as the measurement of gene expression, calculated by the optical density of the treatment group divided by that of control group. The data were analyzed by ANOVA and Dunnet's t test with a significance threshold of 0.05.

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Conceived and designed the experiments: J.B.C., H.W.G., Y.L.Z., X.F.Z., Y.Z. Performed the experiments: J.B.C., H.P.G., Y.Z., C.Q.L. Analyzed the data: J.B.C., H.W.G., Y.Z., Y.L.Z., X.F.Z. Contributed reagents/materials/analysis tools: Y.L.Z., H.W.G., C.Q.L. Wrote the manuscript: J.B.C., H.W.G., Y.Z., H.P.G.

Additional information

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