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Valproic Acid Induces Zebrafish Embryonic Developmental Defects by Inducing Oxidative Stress-mediated Apoptosis: Dose and Time-dependent Analysis

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Abstract

The study aimed to reveal the developmental deformities of the anticonvulsant drug valproic acid (VPA) in zebrafish embryos. The zebrafish embryos were exposed to VPA (up to 100 µM) after 4 hours post-fertilization (hpf) and examined for *in-vivo* toxicity by hatching rate, survival rate, heart rate, oxidative stress, and apoptosis. The VPA has dose and time-dependently affected embryos' hatching, survival, and heart rate. The VPA delayed hatching and noticed unusual hatching at 72 hpf. Complete death of embryos was noticed at doses of 40, 60, 80, and 100 µM VPA at 72 and 96 hpf. Furthermore, VPA has negatively affected the heart rate and was found to be depleted with the dose and time of exposure to VPA. The VPA has induced various developmental defects in embryos, including yolk sac edema, pericardial edema, spinal cord curvature, and tail deformities. DCFH-DA staining revealed that the VPA escalated ROS molecules and induced oxidative stress in embryos. Acridine orange (AO) staining revealed that VPA causes toxicity in embryos by apoptosis. Overall, the study concluded that VPA induces developmental defects in zebrafish embryos by oxidative-stress-mediated apoptosis in dose and time-dependent ways. Thus, our study suggests that VPA release into aquatic ecosystems needs to be limited.

Keywords: Valproic acid, Zebrafish, Developmental toxicity, Reactive Oxygen Species, Apoptosis.

Introduction

To preserve human and animal health, pharmaceutical consumption is expanding internationally. About 4000 compounds are mainly used for human and animal medicine. Large-scale pharmaceutical use poses potential environmental risks (1). Feces and urine excrete medicinal substances as unabsorbed forms and metabolites/byproducts. Pharmaceutical chemicals enter aquatic habitats through sewage discharges, landfill leaching, indiscriminate hospital and residential waste disposal, and stormwater runoff. Drug-resistant infections, infertility, cancer, endocrine disruption, and plant and animal development retardation can result from ng/L levels of hazardous residues. Pharmaceutical residues, such as non-steroidal anti-inflammatory medications, hormones, antibiotics, antiretrovirals, lipid regulators, and β-blockers, adversely damage aquatic ecosystems and human health. Surface water, reclaimed wastewater, and groundwater often contain ampicillin, sulphathiazole, carbamazepine, penicillin, aspirin, paracetamol, amoxicillin, diclofenac, anticonvulsants, vancomycin, efavirenz, and ibuprofen (2, 3, 4).

For more than thirty years, the anticonvulsant drug valproic acid (VPA) and its derivatives have been used to treat epilepsy because they are non-specific inhibitors of histone deacetylase. VPA works by blocking the metabolism of γ-aminobutyric acid (GABA) and interfering with GABA reuptake at nerve terminals. As a result, VPA is used to treat several mental illnesses, including epilepsy, schizophrenia, bipolar disorder, and migraine. Despite being widely regarded as safe, VPA can have serious side effects during therapy, such as significant bone loss (5). Moreover, VPA is cautiously recommended by National Institute for Health and Care Excellence (NICE) for children, young people, and adults with Idiopathic Generalized Epilepsies (IGEs) and pregnant women (6).

The VPA has been detected in municipal sewage and surface water samples, and potentially poses an environmental and health risk. The majority of VPA drugs are taken orally, and 30% to 50% are eliminated as metabolites. Uncontrolled disposal and poor wastewater treatment release VPA's unchanged/unabsorbed form and metabolites/ byproducts into waterways. Due to its limited degradation potential, constant release, and widespread use, VPA may bio-accumulate in aquatic environments while being removed at low concentrations (ng/L or sub-parts-per-billion) (7, 8, 9). VPA accumulation, destiny, and chronic exposure can alter base-of-food-chain biotic populations. Top-of-the-food-chain creatures like larger fishes may bioaccumulate these, generating somatic mutations and systemic toxicity that can cause cancer, biodiversity loss, and death. Moreover, VPA entering the human food chain may cause baldness, elevated alanine aminotransferase and aspartate aminotransferase in the liver, tinnitus, myalgia, and dyspnea. High doses of VPA can cause liver damage, hallucinations, hypothermia, murder, hyponatremia, schizophrenia, toxic epidermal necrolysis, Stevens-Johnson syndrome, allergic reactions, anaphylaxis, syndrome of inappropriate antidiuretic hormone secretion (SIADH), pancreatic inflammation, and allergic reactions. Concentrations can cause thrombocytopenia, pancytopenia, hyperammonaemia, myelosuppression, aplastic anemia, hemorrhage, erythema multiforme, polycystic ovarian syndrome, cerebral pseudoatrophy, encephalopathy, and coma (5, 6). These harmful effects of VPA are known to be caused by the activation of oxidative stress and inflammatory processes. Therefore, antioxidants and anti-inflammatory medications are being extensively investigated in current research as possible therapeutic strategies against VPA damage (10, 11, 12, 13, 14).

We need to gain more knowledge of the impact of VPA on aquatic animals. Therefore, the VPA effect on zebrafish embryonic development was studied in the present study. The reason for selecting zebrafish as a model fish for this study is that they are well suitable for phenotypic screening, embryos are transparent to observe phenotypic changes, maintenance is easy, offspring count is very high, they reproduce all over the year, embryonic development is rapid (15). Research on vertebrate gene function and human genetic illness has been conducted using zebrafish. A high-quality sequence assembly of the zebrafish genome, spanning 26,000 protein-coding genes, has been sequenced, providing insights into the relationship between zebrafish genes and human genes, with 70% of human genes having a zebrafish orthologue (16).

Our study includes toxicological analyses of VPA, including hatching rate, developmental deformities, survival rate, heart rate, oxidative stress (ROS generation), and apoptosis (acridine orange staining).

Materials and Methods

Chemicals and reagents

2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA), VPA, pure water, and acridine orange (AO) were obtained from Sigma-Aldrich, Bengaluru, India. The other

chemicals used in the study were obtained from Merck, Bengaluru, India.

Zebrafish husbandry

Wild-type adult male and female zebrafish were purchased from the local aquarium, Coimbatore, Tamil Nadu, India. The fish were thoroughly inspected for illness and infection. The fishes were maintained in a recirculating system with a 12-hour light and 12-hour dark photoperiod at 27 ± 1°C. The fish were fed with commercially available fish food twice a day. The debris and the tank were cleaned every day and once a week, respectively. The spawning was carried out with 1:2 (male and female zebrafish), and fertile eggs were used for the study (17).

Experimental design

The exposure of zebrafish embryos to VPA was carried out in accordance with FET guidelines (17). The VPA was treated to fertile embryos after 4 hrs post fertilization (hpf). VPA stock solution was prepared in pure water (500 µM) and stored in darkness at -20°C. Then, VPA stock was subjected to dilution to obtain test concentrations of up to 100 µM VPA in an E3 medium for toxicological evaluation. Each experimental group contains 25 embryos. The experiment was carried out at 27 ± 1°C with aeration. The embryos treated alone with the E3 medium were control. The embryos were observed for developmental deformities, survival rate, heart rate, oxidative stress (ROS generation), and apoptosis (acridine orange staining). The observations were noticed at 24, 48, 72, and 96 hpf (18, 19).

Assessment of survival rate and developmental defects

Following the treatment with VPA, as explained in the experimental design. The survival rate and developmental defects of embryos were recorded at specific periods: 24, 48, 72, and 96 hpf (19). The results were expressed as percentages. The observed developmental deformities include yolk sac edema, pericardial edema, somite formation defects, spinal cord curvature, and tail deformities. The developmental deformities were observed under the inverted microscope (EVOS, FLC, Thermo Fisher Scientific, USA).

Assessment of cardiotoxicity

Following the treatment with VPA, as explained in the experimental design. The zebrafish embryos were observed for cardiotoxicity under the inverted microscope. The heartbeat rate was measured at 24, 48, 72, and 96 hpf after the embryos were exposed to the VPA. The heartbeat rate of embryos was measured and expressed per minute (20).

Assessment of oxidative stress by DCFH-DA staining

Following the treatment with VPA, as explained in the experimental design. The effect of VPA on ROS generation in zebrafish embryos was determined by DCFH-DA staining (21, 22). Briefly, before DCF-DA staining, embryos were washed with embryonic media, and staining was carried out at 5 µM of DCFH-DA in water for 15 min in the dark. Following, the embryos were washed thrice with embryonic media, anesthetized, and fixed on glass slides for observation. The green fluorescent protein (GFP) images were captured using the fluorescent inverted microscope (EVOS FLC, Thermo Fisher Scientific, USA). Furthermore, a fluorescent intensity, which reflects ROS levels, was quantified at excitation and emission of 485 and 525 nm, respectively. The results were expressed as percentages with respect to the control group.

Assessment of apoptosis by AO staining

Following the treatment with VPA, as explained in the experimental design. The role of VPA on apoptosis in zebrafish embryos was observed by acridine orange (AO) staining (20). Briefly, following the VPA treatment, embryos were washed twice with water and stained with

5 µM of AO for 15 min in the dark. Following, embryos were washed thrice with embryonic media, anesthetized, and fixed on glass slides for observation. The images were captured using a fluorescent inverted microscope (EVOS FLC, Thermo Fisher Scientific, USA). A GFP filter captured the AO images. The fluorescence intensity of AO was measured at excitation and emission of 485 and 520 nm, respectively. The results were expressed in percentage with respect to the control group.

Statistical analysis

The experiments were carried out independently and in triplicates (n = 3). The results were expressed in mean ± standard deviation. The data was processed by one-way ANOVA, and statistical significance was considered at $p \leq 0.05$. The statistical difference was calculated using Dunnett's test. The analysis and graphical representation were done using GraphPad Prism version 8.

Results and Discussion

Effect of VPA on the hatching rate, survivability, and developmental defects

In the present study, the effect of VPA on the hatching rate, survivability, and developmental defects of zebrafish embryos was assessed at 20, 40, 60, 80, and 100 µM for 24, 48, 72, and 96 hpf. The VPA has been shown to negatively affect the hatching rate of zebrafish embryos (Fig. 1). At 20 µM VPA, hatching of zebrafish embryos was noticed at 48, 72, and 96 hpf. The hatching rate was delayed at 20 µM VPA compared to the control ($p \leq 0.05$). On the contrary, hatching was found completely absent at 60, 80, and 100 µM VPA. The VPA has shown a toxic effect on zebrafish embryos and has affected the survivability of zebrafish embryos, which was found to be dose-dependent and exposure-dependent (Fig. 2). Higher zebrafish embryo death was noticed at a higher tested dose of 100 µM VPA in 24 hpf. In 48 hpf, the complete death of zebrafish embryos was noticed at 80 and 100 µM VPA, and a higher death was at 60 µM VPA. In 72 and 96 hpf, complete death of zebrafish embryos was noticed at a tested concentration of 40, 60, 80, and 100 µM VPA, and a higher death rate was noticed at 20 µM VPA. Thus, the study proved that VPA has affected the survivability of zebrafish embryos and induced death in dose-dependent and time-dependent ways. Moreover, our study observed that VPA had induced various developmental defects in zebrafish embryos at 24, 48, 72, and 96 hpf (Fig. 3). The observed developmental defects include yolk sac edema, pericardial edema, spinal cord curvature, and tail deformities (23). The high concentration of VPA showed a higher intensity of malformation and dead embryos than the low concentration of VPA. In support of our report, Wang et al. have proved that up to 50 µM VPA causes the death of embryos and is responsible for neurotoxicity (20). The results of our investigation demonstrate the substantial toxic effects of VPA on zebrafish embryos, including embryonic death, delayed hatching, and apparent cellular damage in embryos. The study revealed that the toxic effects of VPA rely on the dose and exposure time. This information explains the VPA drug's toxic aberrations and detrimental effects on zebrafish embryonic development.

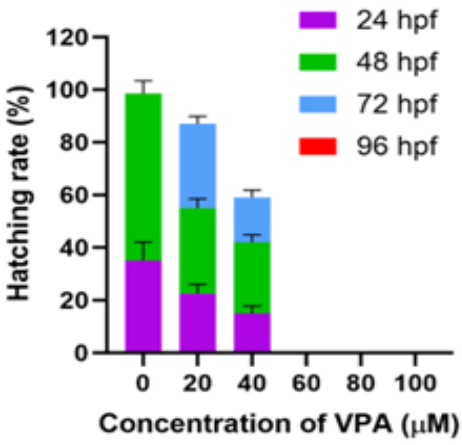

Figure 1: Effect of different concentrations of valproic acid (VPA) on hatching rate (%) of zebrafish embryos at 24, 48, 72, and 96 hpf.

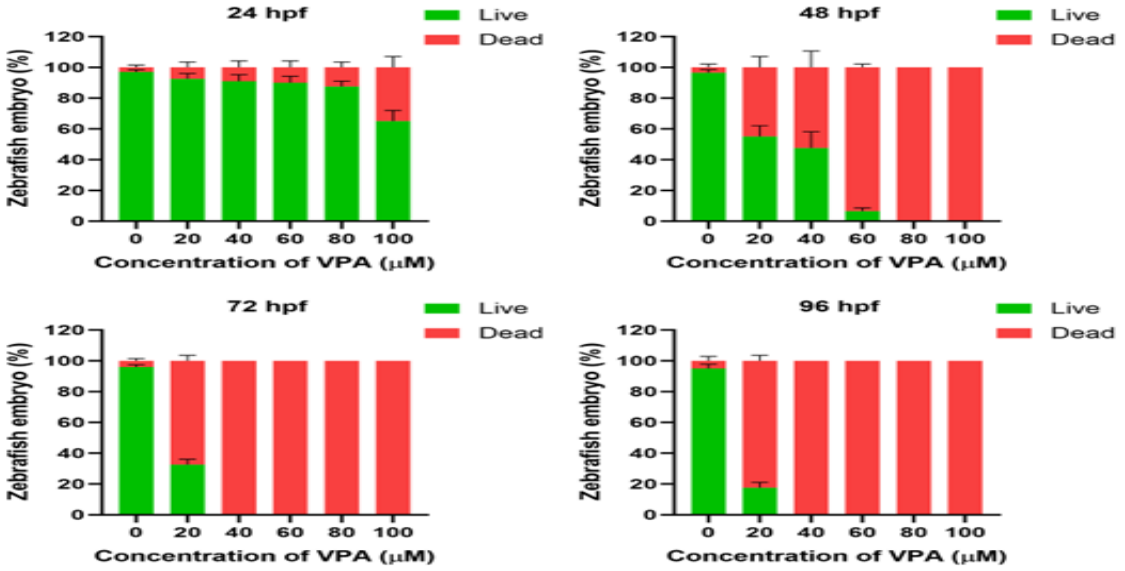

Figure 2: Effect of different concentrations of valproic acid (VPA) on survivability of zebrafish embryos at 24, 48, 72, and 96 hpf.

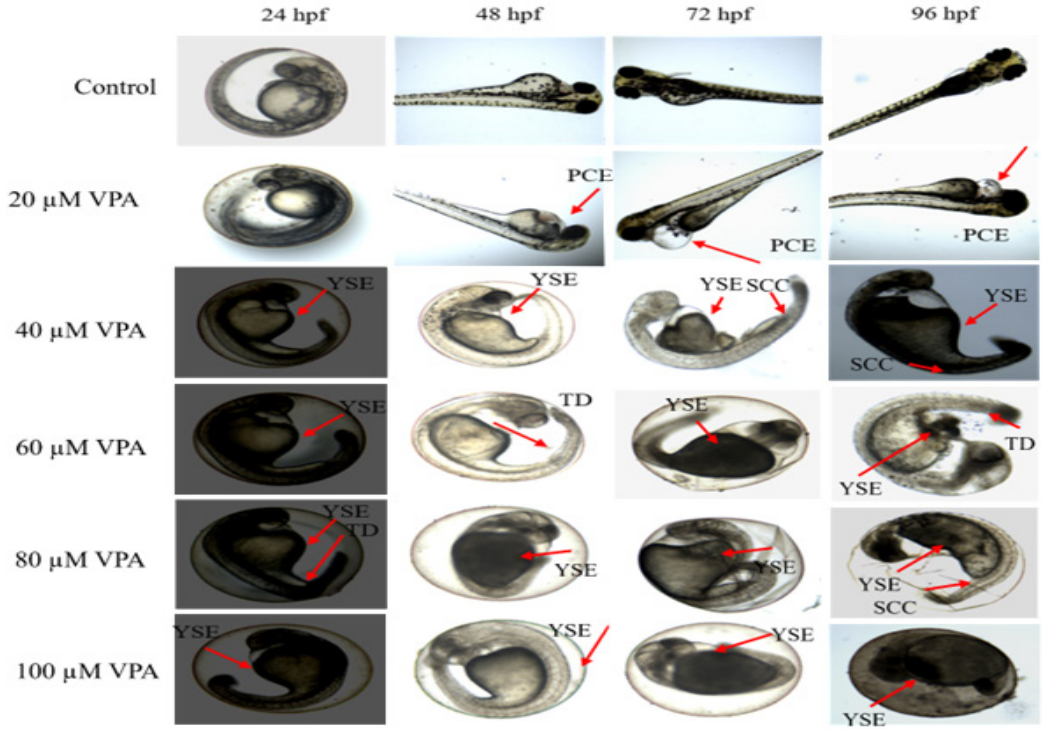

Figure 3: Effect of different concentrations of valproic acid (VPA) on developmental defects of zebrafish embryos at 24, 48, 72, and 96 hpf. YSE: yolk sac edema. PCE: pericardial edema. SCC: spinal cord curvature. TD: tail deformity.

Valproic acid induces zebrafish embryonic developmental defects by inducing oxidative stress-mediated apoptosis: dose and time-dependent analysis

Effect of VPA on heart rate

The zebrafish heart's resemblance to the human heart renders zebrafish a distinctive and essential model for genetics and drug-induced heart failure. The typical zebrafish embryo exhibits a heartbeat ranging from 120 to 180 beats per minute. An accelerated or decelerated heart rate will lead to embryonic death (24). Figure 4 depicts the heart rate of zebrafish embryos subjected to VPA treatment at 24, 48, 72, and 96 hpf. VPA exhibits a significant ($p \leq 0.05$) reduction in heart rate, with values below 80 bpm, compared to the control group. Embryos were found live at 20 µM of VPA, and a heart rate was recorded for 24, 48, 72, and 96 hpf. However, all embryos were found dead at 72 and 96 hpf at 40 and 60 µM of VPA, and the heart rate was not noted. Similarly, all embryos were found dead at 48, 72, and 96 hpf at high dosages of 80 and 100 µM VPA. In support of our report, Wang et al. proved that VPA induced developmental toxicity in zebrafish embryos by dysregulating the heart rate (20).

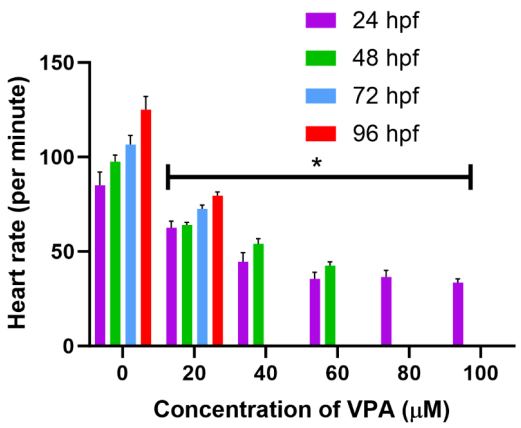

Figure 4: Effect of different concentrations of valproic acid (VPA) on heart rate (per min) of zebrafish embryos at 24, 48, 72, and 96 hpf. The statistical significance between the VPA-untreated (control) and VPA-treated groups was assessed by Dunnett's test. The significance was determined at $p \leq 0.05$ (*).

Effect of VPA on oxidative stress and apoptosis

One of the potential results of toxicity by pollutants is apoptosis. Apoptosis often occurs when a certain intracellular signaling pathway is triggered or when the cell is unable to repair DNA changes. This is the rationale behind the research that simultaneously examines apoptosis and ROS-mediated oxidative stress. In certain situations, ROS molecules harm the nucleus of cells in a way that triggers these proapoptotic signals (25, 26).

In the present study, the effect of VPA on the generation ROS molecules was assessed by DCFH-DA staining and apoptosis by AO staining in zebrafish embryos at 48 hpf (Fig. 5). The DCFH-DA is a highly selective stain for quantifying the ROS molecules and its fluorescence directly related to ROS levels (27). Figure 5A depicts that VPA has dose-dependently escalated DCFH fluoresce levels up to the tested 60 µM VPA compared to the control. The DCFH-DA staining revealed that VPA has dose-dependently escalated the ROS levels up to the tested dosage of 60 µM VPA and was found significant compared to the control ($p \leq 0.05$) (Fig. 5B). Thus, the study concluded that VPA is responsible for ROS-mediated oxidative stress. In support of our study, Wang et al. proved that VPA causes toxicity in zebrafish embryos by ROS-mediated oxidative stress (20).

Similarly, in our study, VPA's effect on apoptosis was revealed by AO staining. The intensity of AO fluorescence directly reflects the apoptosis level. Figure 5A depicts that VPA has dose-dependently escalated AO fluoresces levels up to the tested 60 µM VPA compared to the control. The AO staining revealed that VPA has dose-dependently escalated the apoptosis levels up to the tested dosage of 60 µM VPA and was found significant compared to the control ($p \leq 0.05$) (Fig. 5B). In support of our study, Wang et al. proved that VPA causes toxicity in zebrafish embryos by apoptosis through AO

staining (20). Overall, our study revealed that VPA is responsible for toxic effects in zebrafish embryos caused by oxidative-stress-mediated apoptosis.

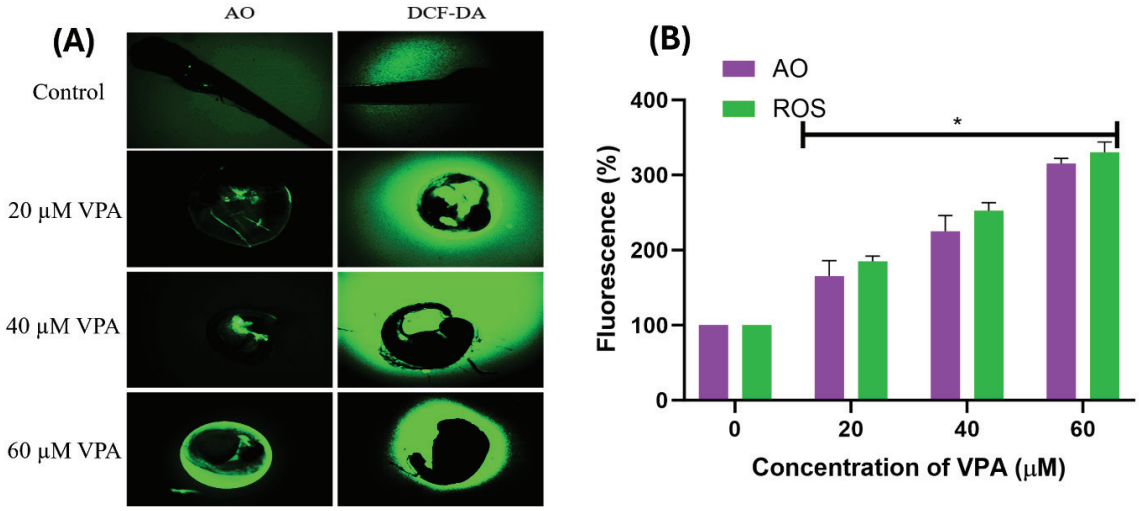

Figure 5: Effect of different concentrations of valproic acid (VPA) on apoptosis by AO staining and reactive oxygen species by DCFH-DA staining in zebrafish embryos at 48 hpf. The statistical significance between the VPA-untreated (control) and VPA-treated groups was assessed by Dunnett's test. The significance was determined at $p \leq 0.05$ (*).

Conclusion

` The study evaluated the toxic effect of the VPA on the aquatic model organism Zebrafish. VPA significantly affected the hatching rate of zebrafish embryos and induced developmental defects of zebrafish embryos. The VPA has lowered the heartbeat rate and caused cardiotoxicity. Moreover, the study showed that VPA elevated oxidative stress by causing excessive ROS accumulation. The possible mechanisms of oxidative stress and apoptosis in VPA-induced developmental defects and cardiotoxicity are elucidated in this work. Even so, substantial molecular investigations must be considered to examine the toxicological effects of VPA. Nevertheless, the relationship between VPA and different organ toxicities must thus be ascertained by assessing the toxicity at various stages of zebrafish development. Consequently, our research shows that VPA is detrimental to aquatic vertebrates during their embryonic phases, could enter the food chain, and exhibit risks to humans. Consequently, the release of VPA into aquatic environments must be constrained.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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