In vivo toxicity of industrial biocide containing 2,2-Dibromo-3-nitrilopropionamide in adult and zebrafish larvae

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Abstract

This experimental study examined the effects of a biocide containing 2,2-dibromo-3-nitrilopropionamide (DBNPA) on 120 fertilized eggs and 77 adult zebrafish. The embryos and adults were exposed to a control group and incremental concentrations of DBNPA. The LC50 was determined after exposure and the data were analyzed by probit analysis. In the 5 and 10 ppm dilutions, some symptoms such as spinal deformity, lack of eye formation, and lack of heartbeat indicated the developmental alterations induced by DBNPA in zebrafish larvae. At a dilution of 100 ppm, all eggs died, showing the high toxicity of this concentration. However, no adverse effects on the growth and health of the larvae were observed in lower dilutions. Regarding hatchability, there was no difference between the 0.1 ppm dilution and the control group, but the hatching rate of eggs decreased with increasing DBNPA concentration.

1. Introduction

Biocides are chemicals that can kill, inhibit, neutralize, or control harmful organisms(Union, 2012). They are widely used in homes and food as cleaners or preservatives. Biocides are meant to fight harmful microbes but can also affect human health if used incorrectly. However, there is not enough toxicological information for the safe use and handling of biocides.(Hahn et al., 2010) Dibromonitrilepropionamide or 2,2-Dibromo-3-nitrilopropionamide is a fast biocide that is easily hydrolyzed in water under both acid and base conditions. It quickly degrades and turns into different products depending on how it decomposes, such as ammonia, bromide ion, dibromoacetonitrile, and dibromoacetic acid. Dibromo Nitrile Propionamide works like other halogenated biocides. DBNPA is a common biocide that is electrophilic and is used in amounts of 0.0002% to 0.02% in paper mills, cooling water systems, heat exchangers, and lab equipment. DBNPA is also used in fracturing fluids. However, some of the biocides that are injected should come back as conversion products. DBNPA could be used as a preservative in industrial glues up to 0.2%(Hwang et al., 2021). If DBNPA were released into the water ecosystem, the amounts would be much lower than when used as a biocide. There is enough data on the possible toxic effects of DBNPA and how workers are exposed to it to assess the risks to humans. In studies of acute toxicity, DBNPA is moderately toxic when swallowed or breathed in (toxicity category II) and slightly toxic when touched by the skin (toxicity category III). DBNPA can damage the eyes (toxicity category I). DBNPA can cause skin allergies. DBNPA seems to be a toxin that affects development in rabbits; changes in structure were seen at a dose that did not harm the mother. Several human incidents related to acute exposure to DBNPA after spilling or misusing it have reported irritation of the eyes, throat, and respiratory system, as well as runny nose or headaches.**(RED)**

In the late 1960s, George Streisinger initiated a search for a vertebrate model organism that could be suitable for elucidating genetics as well as modeling human diseases effectively, a characteristic that most invertebrates lack. **(Grunwald & Eisen, 2002)** Zebrafish have been extensively utilized as a successful animal model in environmental toxicology due to several beneficial traits such as small size, ease of rearing and maintenance, short life cycle, high fecundity, and genetic homologies to humans.**(De Marco et al.,**

2022) Currently, the employment of laboratory animals in toxicology tests is deemed unethical due to the large number of chemical compounds, thus the use of New Approach Methods (NAMs) is favored. Zebrafish embryo toxicity test (ZET) is one of the alternative methods widely used for evaluating developmental toxicity in laboratory conditions to investigate chemicals.**(Fang et al., 2022)** According to European regulations, ZET is not an animal experiment if the zebrafish embryos used in the experiment do not exceed 120 hours post-fertilization (hpf). Therefore, ZET can be used for exploring numerous chemical compounds without violating ethical principles.**(Directive, 2010)** The transparency of zebrafish larvae makes them ideal for studying pollutants that have color or fluorescence**.(Batel et al., 2018)**The ZET is a method that exposes zebrafish to chemicals throughout their larval development, allowing a more comprehensive evaluation of the effects of these chemicals during organogenesis than other existing methods. The ZET is also cost-effective, quick and simple to perform, and can screen a large number of hazardous chemicals.**(Rothenbücher et al., 2019)** The purpose of this study was to compare the in vivo toxicity of DBNPA in zebrafish (Danio rerio) at the embryonic and adult stages and to observe the morphological changes that occurred in the larval assay.

2. Materials and method:

The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies (Tveden-Nyborg et al., 2021)

2.1. Chemical

To prepare a 1000 mg L^{-1} (ppm) solution, 0.1000 gram of the sample supplied by Rosobgiri company (RSCO) was added to 100 mL of distilled water and the remaining dilutions were prepared from the 1000 ppm solution. Dilutions of 0.1, 1, 2, 5, 4, 6, 8, 10, 50, 75, and 100 ppm were chosen to perform this test.

2.2. Adult zebrafish test

Seventy-seven adult zebrafish, about 5 months old and about 1-2 cm in size, were obtained from the zebrafish laboratory of the Department of Toxicology, Faculty of Veterinary Medicine, University of Tehran, Iran and maintained in system water at a temperature of 26°C for 9 days. Of 77 zebrafish, 39 were male and 38 were female, both sexes were represented in different test groups. The light-dark cycle with 14 hours light and 10 hours dark was used. Water was also tested for pH, hardness, and saturated oxygen. The conditions used followed OECD-203 Protocol **(OECD, 2019)** . According to OECD, seven fish were selected for each dilution and they were placed individually in a tank containing one liter of system water. According to the protocol, no feeding was carried out during the test. Seven fish in the test tank with system water served as a control group. After placing the fish in the test tank, the fish were observed daily twice a day in the morning and in the afternoon and this was continued for 96 hours. A fish is considered dead when there is no movement and the caudal fin does not produce a response. Mortality was recorded and dead fish were removed as soon as possible. The fish were also observed for abnormal clinical signs such as loss of equilibrium, abnormal swimming behavior, abnormal respiratory function, and abnormal skin pigmentation.

2.3. Zebrafish Embryotoxicity Test

To prepare zebrafish eggs, 2 female fish and 3 male fish were purchased from the zebrafish laboratory of Toxicology Department, Faculty of Veterinary Medicine, University of Tehran, and after checking and confirming their health according to the OECD Protocol 236**(OECD, 2013)** , they were placed in the special spawning tank. After darkness had been induced for 12 hours, the tank emerged from darkness in the early hours of the morning and the separator between male and female fish was removed. Spawning occurred 30-60 minutes after the separator was removed. Eggs were collected with a Pasteur pipette and the eggs were rinsed twice in E3 solution (including 5mM NaCl, 0.17mM KCl, 0.33mM CaCl2.2H2O, 0.33mM MgCl2.7H2O) to remove debris to clean. The test was performed as soon as possible after fertilization. 20 fertilized eggs are transferred to 96-well plates which pre-saturated for 24 hrs and exposed for each concentration (0, 0.1, 1.0, 10.0, and 100.0 ppm and refilled with freshly prepared test solutions within 180 minutes post fertilization. The wells containing exposed and control eggs were kept in the incubator at a constant temperature of 28 degrees. The DBNPA solutions were renewed and embryonic/larval mortality

and hatching rate were evaluated every 24 h. The hatching rate is a ratio of hatching embryos to the reaming living embryos in each well. the eggs were observed daily for 96 hours using an inverted microscope (Zeiss, Germany).

2.4. Statistical analysis

All data analyses were performed using the GraphPad Prism (v 9.5.1) software and SPSS (v 26.0). Determination of LC50 was performed using profit and P-values less than 0.05 were considered statistically significant.

3. Results:

Toxicity tests from the present study resulted in a LC50 of 9.3 ppm for adults and 9.1 ppm for the larvae (data are not given). As can be observed in Figure 1, the morphological changes at the 5 and 10 ppm dilution are collected in one photo, in this groups, egg coagulation, no eye formation, non-detachment of the tail, yolk sac edema, spinal curvature and delayed egg hatching at different hours after fertilization were evident. As expected, at a dilution of 100 ppm, all the fertilized eggs were coagulated on the first day after fertilization (figure 1).

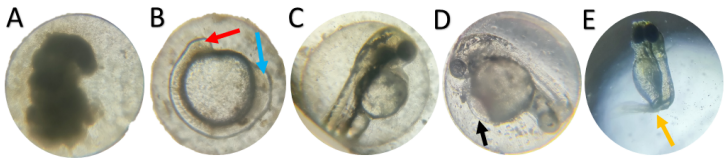

Figure 1 – Malformation of zebrafish embryos exposed to different concentration of DBNPA solutions. (A) coagulated embryo at 100 ppm (B) non-detachment of the tail (red arrow) and lack of eye formation (blue arrow) at 10 ppm (C) delay hatching after 72 hours at 10 ppm (D) Yolk sac edema (black arrow) at 5 ppm (E) skeletal malformation (orange arrow) at 10 ppm.

In the control group (E3), 0.1 and 1.0 ppm no morphological changes were observed, the eggs and larvae were completely normal up to 96 hpf. Larvae normality was confirmed according to the 236-OECD protocol by non-coagulation of eggs, somite formation, tail detachment, and the presence of heartbeat. (Table 1)

Toxicity	Time (hpf)	C (ppm)	C (ppm)	C (ppm)	C (ppm)	C (ppm)	C (ppm)
		0	0.1	1	5	10	100
Coagulated embryo	24	0	0	0	3	8	20
Lack of somite formation	48	0	0	0	0	0	-
Non detachment of tail	72	0	0	0	0	2	-
Lack of heartbeat	96	0	0	1	0	1	-
Egg hatching	72	19	19	17	11	10	0

Table 1 – Zebrafish embryo toxicity test result according to oecd-236

When examining the rate of egg hatching in 72 hours after fertilization in the control group and 0.1 ppm 19 eggs, in the 1 ppm group 17 eggs, in 5 ppm 11 eggs, and in the 10 ppm 10 eggs out of 20 eggs were hatched (figure 2).

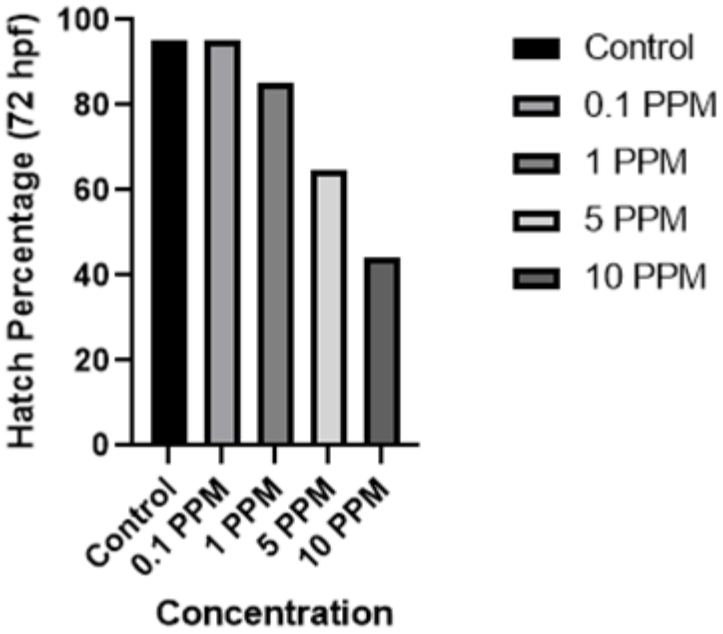

Figure 2 – zebrafish egg hatching percentage at different groups of exposure

According to figure 3-A, seven fish were tested at different dilutions and the results of 96 hour studies can be viewed. At higher dilutions the mortality rate was much higher, such that at a dilution of 100 ppm all fish died in less than 30 minutes but at a dilution of 10 ppm losses were observed 24 hours after exposure. In the 10 ppm group, the fish showed over-reactive to stimulus but in the 50 ppm and 70 ppm groups, the decrease in spontaneous activity was evident. In the 100 ppm group, two fish showed irregular opercular ventilatory movements. At the dilutions of 0.1, 1, 2, 4, 6 and 8 ppm in the test relative to adult fish, no suspicious symptoms were evident after 96 hours of exposure and by the end of the test all fish were completely normal.

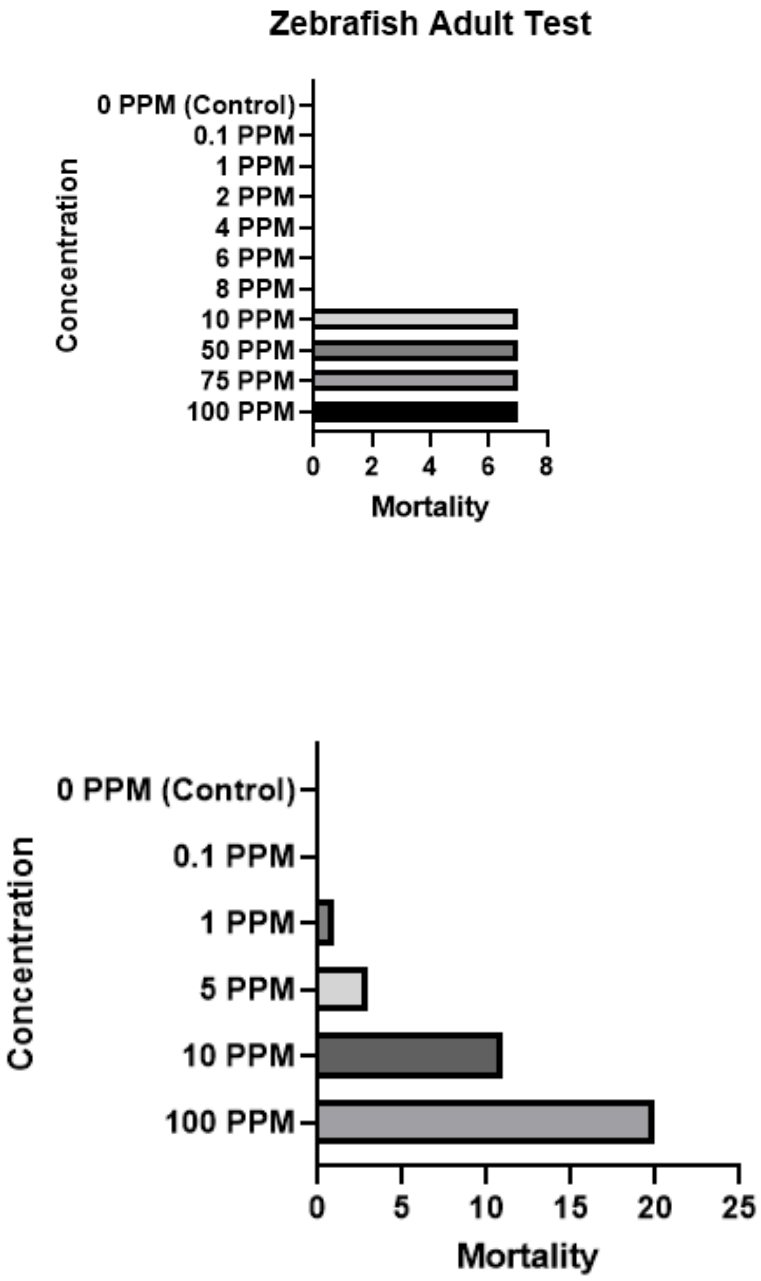

Figure 3 – A) Mortality rates in adult zebrafish exposed to different dilutions of DBNPA B) Mortality rates in zebrafish larvae exposed to different dilutions of DBNPA

4. Discussion and Conclusion:

Our study is similar to some previous studies that used zebrafish as a model organism to assess the acute toxicity of various chemicals. For example, Ali et al. (Ali et al., 2011) used zebrafish embryos to determine

the LC50 values of 60 drugs and compared them to the LD50 values of rodents. They found a strong correlation between the two endpoints and suggested that zebrafish embryos could be a useful predictive model for toxicity testing. Ducharme et al. (Ducharme et al., 2015) used zebrafish embryos to compare toxicity levels across mammalian studies and databases. They found good agreement between zebrafish embryo LC50 values and mammalian oral LD50 values, but poor agreement with mammalian intravenous LD50 values. They also identified some chemical classes that were more toxic to zebrafish than mammals, such as carboxylic acids, glycosides and alkaloids. The results of this study showed no significant difference in the amount of LC50 calculated from adult zebrafish and zebrafish larvae, but in the test related to zebrafish larvae, the presence of signs of developmental toxicity was significant. Considering the short lifespan of zebrafish, examining the effects of common chemicals in different industries on zebrafish of different ages can give us a good overview of the effects of using different chemicals throughout life. On the other hand, due to the lack of significant differences in the LC50 values obtained in both the adult and zebrafish larvae tests, the zebrafish larvae test, which is more ethical, can be used for many available chemicals.

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