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Research Article

Effects of Heavy Metals on the Embryo and Larvae of Zebrafish, *Danio rerio* (Cyprinidae)

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Abstract: Toxicity tests using early life stages of fish are of great importance in assessing risks to growth, reproduction and survival in polluted environments and are important tools for good environmental monitoring. The present study deals with the effect of heavy metals (zinc, nickel and chromium) on the embryo and larvae of zebrafish, *Danio rerio*. Embryo were exposed to Zn (10, 20, 30, 40, 50 mg/l), Ni (40, 50, 60, 70, 80 mg/l) and Cr (7, 12, 17, 22, 27 mg/l) however, larvae were exposed to 4, 9, 14, 19, 24 mg/l Zn, 15, 20, 25, 30, 35 mg/l Ni and 3, 6, 9, 12, 15 mg/l Cr. The 72-h LC_{50} value for embryo is 41.08 mg/l for Zn, 69.78 mg/l for Ni and 15.99 mg/l for Cr. The 24 to 96-h LC_{50} value for the larvae showed a gradual decrease as the exposure time was increased. The 96-h LC_{50} for larvae is 12.01 mg/l for Zn, 31.13 mg/l for Ni and 6.24 mg/l for Cr. The toxicity was concentration as well as time dependent. Toxicity tests revealed that larvae of zebrafish are more sensitive to heavy metals as compared to the embryos. The probable causes have been discussed.
Keywords: Embryo, Larvae, Mortality, Fish, Heavy metal

INTRODUCTION

Trace metals are natural components of the biosphere and some metals are essential for life but their excess concentrations become toxic. These metals are introduced into the environment by a wide spectrum of natural and anthropogenic sources. The main sources of heavy metal pollution are industrial effluents. The indiscriminate discharge of heavy metals in water bodies lead to ecological degradation and lethal effects on non-target organisms. They disrupt the food chain threatening the ecological balance and the biodiversity of the nature. They are highly toxic to fish and other aquatic organisms in highly polluted water, as is indicated in residues of fish [1]. Toxicity test of chemicals on animals has been used for a long time to detect the potential hazards posed by chemicals to man. The reduced fitness and growth of fish occurs at sub-lethal levels depending on exposure time, toxicity and concentration of the chemical substances involved. The reproductive ability and early life stages of fish, like eggs and larvae are particularly sensitive to contaminants [2]. The fact is that metals are non bio-degradable and can accumulate in the environment, make them deleterious to the aquatic organisms and consequently to human being who consume fish as a food source.

Animal embryos in particular had been recognized as a valuable and cost effective tool for monitoring water quality because this life-stage generally responds quickly even at lower toxicant concentration [3]. The developing fish embryos/larvae are generally considered to have the highest susceptibility in the fish cycle. Thus, it is relevant to use fish embryos or larvae as biological indicators for determining the water quality criteria. Among freshwater animals, zebrafish, *Danio rerio* are widely used for vertebrate embryo toxicity bioassays [4-6]. For the study of embryonic development, toxicologist began searching for the alternative methods to test chemicals, because under the current guidelines such testing would have required millions of laboratory animals [7]. Earlier we observed the toxicity of Dimethoate on adult, embryo and fingerlings of zebrafish which caused the significant reduction in fecundity, viability and survival of fingerlings [8].

Zebrafish spawns every 1-6 days during spawning season. The female releases 5 to 20 eggs at a time. This cycle repeats for about an hour. The hatchlings (3mm) are able to swim, feed and exhibit active avoidance behaviors within 72-h of fertilization. The development of zebrafish is rapid, with precursors to all major organs developing within 36-h and larvae display food-seeking and active avoidance behaviours within five days after fertilization, *i.e.*, 2 to 3 days after hatching. Zebrafish have been widely used for detection of heavy metal contamination, making this animal a convenient biological water contaminant sensor [9]. There are several reports of heavy metals effect on

embryo and larvae of fishes living in polluted water bodies [10, 11]. However there is little information on the effects of Zn, Ni and Cr on life fish development and its potential toxicity at sub-lethal concentrations.

Studies on zebrafish can provide an integrative framework for predicting risk to wild fish populations [12]. The zebrafish was selected as the test species for toxicological studies as per to the recommendation of the Organization for Economic Co-operation and Development [13] for using fish as test organisms for the early life stage toxicity. Zebrafish embryos have some important characteristics such as in vitro fertilization, rapid embryonic development and optical transparency, which make it easy to detect morphological endpoints or observe the development process in early life stages [14]. As an initial step to better understand the potential eco-toxicological impact of heavy metals released into the aquatic environment, a 72-h embryo and 96-h larvae bioassay was used to assess and compare the toxicological effects of Zn, Ni and Cr on zebrafish, *Danio rerio*.

MATERIALS AND METHODS

Collection and maintenance

Zebrafish was recorded for the first time from Uttar Pradesh, was collected from local ponds. They were acclimatized under laboratory conditions in 35-l glass aquaria containing dechlorinated water, aerated continuously through stone diffusers connected to mechanical air compressor. The zebrafish is a hardy fish and can withstand a pH ranging between 7.6-8.5 and temperature maintained between 25 ± 2^0C. During the acclimation period, the adult fish were fed twice per day with brine shrimp and egg albumin. The care and husbandry of zebrafish used in this study was in conformity with the guidelines [15] that regulate the humane care and use of laboratory animals for research purposes.

The zebrafish we used as spawners had a length of 3.62±0.04 cm and 1.00±0.48 g weight. Prior to spawning, males and females were housed separately for a minimum of 5 days. The day before eggs were required, males and females were taken in the ratio of 2:1 (male: female). Eggs were obtained by placing nylon net between the fish and the bottom of the aquarium. Spawned eggs fell through the net, thus preventing the adult fish from eating the eggs. Two hours after oviposition eggs were transferred to petridish and checked for fertilization under a dissection microscope. Opaque, non-fertilized eggs and inactive embryos were removed.

Test substances

$NiCl_2.6H_2O$, $K_2Cr_2O_7$ and $ZnCl_2$ (Merck®) were dissolved in distilled water in order to prepare the stock solution.

Toxicity test

It was done by the method of Ansari and Kumar [16]. For the bioassay of embryo and larvae, the fertilized eggs of zebrafish were obtained by mass breeding in the laboratory. Embryo and larvae of zebrafish were exposed to various concentrations of heavy metals. Each experiment was accompanied with their respective controls (without heavy metals). Water was continuous aerated and renewal of the water occurred in a semi-static manner (complete renewal of solution after 24 h).

For embryo toxicity, 300 fertilized eggs were separated in 500 ml beakers with 250 ml dechlorinated water. These eggs were exposed to the selected five different concentrations of Zn (10, 20, 30, 40, 50 mg/l), Ni (40, 50, 60, 70, 80 mg/l) and Cr (7, 12, 17, 22, 27 mg/l) for 72-h. Since, the complete embryo is almost formed after 72-h, hence the eggs having embryo were exposed only up to 72-h. The dead embryos became white due to coagulation or precipitation of protein.

To perform the larvae toxicity, the eleutheroembryonic stage *i.e.*, the 5-day old free-swimming larvae of zebrafish was used. Thirty larvae were used, to evaluate the toxic effect of the heavy metal. By trial and error method the maximum concentration for 100% mortality and minimum concentration for 0% mortality was calculated. Finally, five different concentrations of each heavy metal were selected for the toxicity test. For Zn 4, 9, 14, 19, 24 mg/l, for Ni 15, 20, 25, 30, 35 mg/l and for Cr 3, 6, 9, 12, 15 mg/l were selected. Mortalities of larvae were recorded after 24, 48, 72 and 96-h exposure periods. The dead larvae were removed from the test water.

Data analysis

The susceptibility of the embryos and larvae of zebrafish to Zn, Ni and Cr were determined by the probit method of Finney [17] using StatPlus® version 2009 computer software programme purchased from analystsoft Vacouver, Canada. The lethal concentrations (LC_{10}, LC_{50} and LC_{90}), Confidence limits, Slope and Chi-square values at different exposure periods were established.

RESULTS AND DISCUSSION

During the embryonic stage, the average number of dead embryos increased with increase in the concentrations of the heavy metals (10, 20, 30, 40, 50 mg/l Zn; 40, 50, 60, 70, 80 mg/l Ni; 7, 12, 17, 22, 27 mg/l Cr). LC_{50} value decreased with the increase in time. Chromium was more toxic than zinc and nickel. The order of toxicity was Cr>Zn>Ni. It is evident from the table 1 that the 24-h LC_{50} value for Zn was 92.43 mg/l while for 48-h and 72-h it was 50.02 mg/l and 41.08 mg/l respectively. Similarly, the 24-h LC_{50} value of Ni was 131.91 mg/l while for 48-h it was 92.54 mg/l, which was decreased to 69.78 mg/l after 72h exposure. The 24 h LC_{50} value of Cr was 45.36 mg/l, while for 48

h it was 30.01 mg/l which was decreased to 15.99 mg/l after 72 h exposure period.

In the larvae stage, the numbers of dead larvae were observed at different concentration of heavy metals (Zn, Ni and Cr) at different exposure period (24, 48, 72 and 96-h). The number of dead larvae increased with increase in the concentration of the heavy metals. For Zn 24-h LC_{50} value was 36.95 mg/l which was decreased to 12.01 mg/l after 96-h. For Ni it was 52.88 mg/l, for Cr it was 16.36 mg/l after 24-h which was significantly decreased to 31.13 mg/l and 6.24 mg/l for Ni and Cr respectively after 96-h (Table 2). The highest concentration *i.e.,* 24 mg/l of Zn, 35 mg/l of Ni and 15 mg/l of Cr showed the highest larval mortality. Thus from the result, the order of toxicity, for the larvae was Cr>Zn>Ni.

The treated larvae showed slow movement with tendency to setting down at the bottom of the beaker, motionless for about 25-30 minutes with decrease in opercula beat. It was also observed that the larvae exhibited inconsistent jumping, erratic swimming, loss of balance and incessant of air. The zebrafish larvae in the control exhibited normal swimming behavior.

Table 1: Toxicity of Zinc, Nickel and Chromium to the Embryo of Zebrafish.†

Metals	Exposure period (h)	Effective concentrations (mg/l)			Confidence limits of LC_{50} (mg/l)		Slope value	Chi-square value
		LC_{10}	LC_{50}	LC_{90}	LCL	UCL		
Zn	24	21.65	92.43	394.62	76.07	122.83	4.63	4.33
	48	16.41	50.02	152.52	37.47	113.62	3.56	14.64
	72	13.36	41.08	126.29	23.03	44.17	3.00	21.77
Ni	24	59.19	131.91	293.99	113.08	169.77	2.79	4.06
	48	51.18	92.54	167.31	86.87	100.76	2.74	4.14
	72	45.82	69.78	106.26	68.03	71.77	2.08	4.69
Cr	24	10.49	45.36	196.19	30.76	57.91	4.67	7.02
	48	8.94	30.01	100.70	27.19	34.12	3.84	13.38
	72	7.74	15.99	33.04	11.91	20.97	2.63	19.90

†300 eggs were used for each concentration. Embryos were exposed to Zn (10, 20, 30, 40, 50 mg/l), Ni (40, 50, 60, 70, 80 mg/l) and Cr (7, 12, 17, 22, 27 mg/l).

Table 2: Toxicity of Zinc, Nickel and Chromium to the Larvae of Zebrafish.†

Metals	Exposure period (h)	Effective concentrations (mg/l)			Confidence limits of LC_{50} (mg/l)		Slope value	Chi-square value
		LC_{10}	LC_{50}	LC_{90}	LCL	UCL		
Zn	24	6.77	36.95	201.59	23.97	85.54	5.59	0.12
	48	4.49	29.23	190.19	19.82	71.66	6.42	0.28
	72	3.76	20.16	108.14	15.12	35.23	5.52	0.49
	96	2.77	12.01	52.01	9.16	15.57	4.51	0.62
Ni	24	21.83	52.88	128.07	38.76	104.28	2.98	0.24
	48	16.34	47.54	138.31	35.41	74.91	3.44	0.23
	72	14.52	35.96	89.02	30.01	58.58	3.03	0.13
	96	13.44	31.13	72.09	26.94	41.47	2.88	0.12
Cr	24	4.69	16.36	57.07	12.57	29.86	3.96	0.10
	48	3.19	13.23	54.73	10.29	21.62	4.52	0.14
	72	2.48	8.73	30.76	7.01	11.09	3.98	0.43
	96	2.13	6.24	18.23	4.91	7.49	3.45	0.81

† 30 larvae were used for each concentration. Larvae were exposed to Zn (4, 9, 14, 19, 24 mg/l), Ni (15, 20, 25, 30, 35 mg/l) and Cr (3, 6, 9, 12, 15 mg/l).

Animal Welfare Organization have increasingly questioned ecotoxicity testing with fish and stimulated efforts to develop various alternatives. A promising alternative approach to classical acute fish toxicity testing with live fish is the fish embryo toxicity test (FET) [18], which has been used for the exact evaluation of chemical toxicity to fish [19]. Li *et al*. [20] conducted their experiment on zebrafish and result clearly indicates that sodium arsenite exposed embryos exhibited delayed hatching. Similarly, Scheil *et al.* [21] studied the effect of 3, 4-dichloroaniline and diazinon on zebrafish embryos and larvae and found that they causes impairment in development, locomotor activity and mortality. Also, Ali and Legler [22] studied the effect of Nonyphenol (NP) an industrial organic compound on the embryo of zebrafish and found that embryos treated with NP showed developmental abnormalities and morphological alterations in a dose-dependent manner.

Earlier several other workers also studied the effect of different contaminants on the embryo and juveniles of zebrafish [2, 23, 24]. In the present study, bioassay of zebrafish embryo and larvae was made and it was found that very low concentrations of all the three metals (Zn, Ni and Cr) are effective to kill the 10%, 50% and 90% of the total population, as it is evident from the results (Table 1 & 2). In this study we found that exposure of Zn, Ni and Cr on zebrafish caused increase in the number of dead embryos and larvae. It was also found that the embryos of zebrafish were less sensitive to heavy metals as compared to larvae, due to the presence of chorion. In a recent report Klee *et al*. [25] examined the effects of nicotine on early development of zebrafish. Earlier, it has been reported that zebrafish fingerlings are sensitive to lambda-cyhalothrin and neemgold [26].

Therefore, it can be attributed that the hatching was affected due to the inhibition of some hatching enzymes. The mortality of the larvae was increased with the increase in concentration and exposure periods of the three metals during the present work. Thus, early life stage mortality and decreasing reproductive success are of special importance because their occurrence is not easily detected and alteration in population structures and abundance may only be perceived over the long-term. However, recently it has been also reported that embryos of zebrafish were less sensitive to contaminants as compared to fingerlings, due to the presence of chorion [8].

It is clear from the present study that zebrafish and its early life stages are sensitive to low levels of heavy metals (Zn, Ni and Cr) and significantly affect its populations. From the present study, it may be presumed that the exposure to sub-lethal doses of metals might have caused acetylcholinesterase inhibition, which could drastically affect growth, hatchability, survival, feeding and reproductive success of fishes as reported by other workers [2, 27]. Thus, fishes are not the exception and they may be highly polluted with heavy metals leading to serious problems and ill effects. Therefore, heavy metals should be treated before discharging into aquatic systems.

Kucukoglu *et al.* [28] observed that in 1.0 mg/l zinc chloride, the exposed groups of zebrafish hatching began at 7 days instead of 4 days, and most of the embryos died without hatching at 11 and 12 days. Development deformities such as abnormal embryogenesis, low hatching, delayed hatching and reduction of newly hatched larvae and poor survival ratio was also observed. Also Kienle *et al.* [24] studied the toxicity of nickel chloride on zebrafish embryo and larvae and found that Ni targets the active sites of enzymes and regarded as an unspecific toxicant for aquatic organisms. Abnormal locomotory activity, morphological abnormalities and mortality of embryo and larvae was also observed. Factor and Chavez [29] compared the toxicity of As, Al, Cr and Ni to the embryos of *Radix quadrasi* snail and the order of toxicity was Cr>As>Ni>Al. Kazlauskiene and Vosyliene [30] studied the effect the singly Cu & Zn and Cu-Zn mixture on the rainbow trout, *Oncorhynchus mykiss* and found that hatching embryos had the greatest sensitivity of the effect of metals singly and in the mixture (the highest percentage of mortality was observed). Similarly, Sayed *et al.* [31] studied the effect of 4-nonyphenol and observed the increase in incubation period, mortality rate and malformed embryos ratio, morphological changes and histopathological alterations were also observed.

In conclusion, this study indicates that the presence of heavy metals in aquatic ecosystems can significantly interfere with the distribution of species, mainly due to their high toxicity to fish embryo and larvae. Therefore, early life-stage of zebrafish provides an ideal model for studying the adverse effects of heavy metals. This implies that one should take a necessary precaution in the application of heavy metals to protect fish and other aquatic fauna. It is also suggested that these types of toxicological studies are highly required to monitor the water bodies and assess the toxic effect of heavy metals on non-target organisms particularly the fish. This data would be useful in further studies of heavy metals effect on fish or determining the water quality guidelines for the protection of aquatic biota as well as solving the problems of aquatic toxicology. Therefore, it should be advisable for further research to focus on the effects of long term impacts of heavy metals on zebrafish.

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