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Zebrafish (*Danio rerio*): Effect of Cadmium Chloride on the Developmental Parameters in Embryos and Larvae

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ABSTRACT: Zebrafish (*Danio rerio*) is a good model system for studying different toxicity tests. The reason behind this is that we can compare the genome of Zebrafish with humans, they show 70% similarity with human genes and 84% of genes associated with human disease. Another reason is the short life cycle of the fish and its transparent body. The embryos are also transparent and develop outside the mother. The embryos are used for the experiments because the changes can easily observe due to the presence of any chemical or metal. Cadmium chloride is a very toxic and non-essential heavy metal present in the environment. Due to the presence of this metal different types of development changes were seen in the early stages of embryo development. In the study, different concentrations of cadmium chloride 25, 50, 75, 100, 125 mg/500ml were used and an equal number of embryos was exposed to each concentration. Observations were taken comparing with the control embryo. The changes observed in the cadmium exposed embryos were decreased in some somite's, delay in hatching rate, slow development of pectoral and caudal fin, and decrease in size of the eye, and show no difference in neural mid- hindbrain lining. From the study, it is clear that if the concentration of cadmium is low then no change is observed but as the concentration increases abnormalities in the developing embryos are seen.

KEYWORDS: zebrafish, cadmium chloride, embryos and larvae, somite's, head malformation, fin development, reduced eye lens.

I. INTRODUCTION

Aquatic organisms such as fishes are in most cases exposed to multitudes of stressors that are either natural or anthropogenically introduced into the environment and these stressors can be any type of impurity in the water body like toxic chemicals or heavy metals. Zebrafish (*Danio rerio*) is one of the most used organisms as a model organism in studying different types of disorders or different types of toxicity tests. There have been few studies that attempted to investigate the possible effects of heavy metal toxicity. Toxic metals such as cadmium, mercury, and lead are widely disseminated in the environment because of the extensive use of these metals in industry and consumer products (Elly Suk Hen Chow. et.al 2008).

The Zebrafish (*Danio rerio*) is an important and widely used vertebrate model organism in scientific research, for example in drug development, in particular pre-clinical development. It is also notable for its regenerative abilities and has been modified by researchers to produce many transgenic strains. The approximate generation time for Zebrafish (*Danio rerio*) is three months. Fertilized eggs almost immediately become transparent, a characteristic that makes *Danio rerio* a convenient research model species. The most important reason for selecting zebrafish for the study is that the embryos develop outside the mother. Embryos and larvae are completely transparent so it becomes easy to perform experiments.

The developing embryos are considered to be the most sensitive stage in the life cycle of the fish. It becomes very easy to observe various changes that occur in the embryos and larvae in the developing stages. Previous studies reveal that the sensitivity of fish embryos and larvae to chemicals is far greater than that of an adult. For many contaminants, the sensitivities of Zebrafish embryos and larvae are similar. However, the egg chorion can act as a barrier to protect fish embryos from various contaminants including metals, some polymers, polychlorinated biphenyls, antibiotics, and high molecular weight non-ionic surfactants, resulting in lower sensitivities to waterborne exposures of these contaminants.

Indeed, metal sensitivity is highest during the larval stage, compared to other times in the life history of a fish (Derek Alsop, Chris M. Wood 2011).

Metals are the most toxic substances present in the environment. Due to their widespread dispersal from mining, coal burning, sewage, as well as agricultural, industrial and domestic runoff, they are of global concern. Likewise, if these metals are present in the aquatic environment it becomes a hazard for fishes living in the aquatic body. There are many heavy metals present naturally in the water but some metals are introduced anthropogenically in the water due to these different types of abnormalities seen in the fishes. The metals like cadmium chloride, zinc, nickel, copper, etc. can affect the life cycle of the fishes.

In the study, cadmium chloride is used. Cadmium chloride is one of the most toxic heavy metals present in the aquatic environment. It is a white crystalline compound of cadmium and chlorine, with the formula CdCl_2 . It is a hygroscopic solid that is highly soluble in water.

In this study, the possible defect in Zebrafish embryos upon cadmium exposure was examined. By exposing the embryos and larvae to the different concentrations of cadmium chloride shows morphological changes like the formation of the small eye, deformation of neural lines, reduction in tail formation. Effect on hatching time, slower down the development of fins, movement of the larvae get slow. In lower concentration normal growth and development of organs are seen but as the concentration increases the changes are seen when compared with control embryos and larvae.

II. REVIEW OF WORK DONE ON ZEBRAFISH (*Danio rerio*)

In 2004 A.V.Hallarea, M.Schirlinga *et.al* experiment on zebrafish embryos to determine the interrelation between temperature and cadmium on Zebrafish (*Danio rerio*). In the experiment they use six different concentrations of cadmium (0, 0.25, 0.5, 2.0, 5.0, and 10.0 mg/l) and put them in three different temperature (21°C, 26°C and 33°C) respectively. The embryos were exposed to each concentration and kept them at different temperature. The experiment was performed in triplicate. Observations were done. The result shows the change in mortality rate, development of different organs, decreased heart rate, delay in hatching rate, etc.

In 2008 Elly Suk Hen Chow^b, Michelle Nga Yu Hui *et.al*. Experimented to determine the effect of cadmium on the development of the brain during the early developmental stage. In this study, embryos were continuously exposed to 100 μM cadmium chloride in embryo medium from sphere stage (4 hpf) to end of segmentation stage (24 hpf). Observations were done in an interval of 2 hours. The result of the experiment shows head malformation, embryos typically presented a smaller head with unclear boundaries between the brain subdivisions, particularly in the mid-hindbrain region.

In 2008 the study was performed by Elly Suk Hen Chow, Michelle Nga Yu Hui *et.al*. To determine the effect of cadmium on the development of eyes of Zebrafish (*Danio rerio*). They investigate the cellular basis of the formation of the small eye phenotype and the induction of blindness by studying retina development and retinotectal projections. Embryos were continuously exposed to 100 μM cadmium chloride in embryo medium from the sphere stage (4 hpf) to the end of the segmentation stage (24 hpf). Observations were done in an interval of 2 hours. The result of the experiment shows that exposed embryos exhibited less than 70% of the mean area of the eye, which was classified as the "small eyes" phenotype. Loss of pigmentation in the retinal pigment epithelium (RPE), some larvae were blind. Fish adapt their skin melanin pigment levels in order to blend in with the background as a camouflage response. This response is dependent on visual function as blind fish are hyper pigmented in a well-lit environment. Since Zebrafish embryos suffered from small eyes in response to cadmium exposure.

In 1995 the study was performed by CHARLES B. KIMMEL *et.al* to describe a series of stages for the development of the embryo of the Zebrafish, *Danio (Brachydanio) rerio*. They define seven broad periods of the embryogenesis-the zygote, cleavage, blastula, gastrula, segmentation, pharyngula, and hatching periods. These divisions highlight the changing spectrum of major developmental processes that occur during the first 3 days after fertilization using staging series.

In 2014 the study was performed by Jian YIN, Jian-ming YANG *et.al*. To determine the effect of cadmium sulfate and α -naphthoflavone on Zebrafish (*Danio rerio*). In the experiment both the chemicals were used separately and in combined form. 75mg/l cadmium sulfate was dissolved in the holtfreter's buffer for exposure. Here 24 well plate was

used to expose embryos. In each well, 10 embryos were kept. The embryos were observed at the particular period (24h, 48h, and 72h post-fertilization). The result shows that CdSO₄ treated embryos show both lethal and sub-lethal effects like 24hpf embryos die and 73hpf embryos show delayed hatching. However sub-lethal effects, such as 48hpf show cardiac edema and delayed hatching in 72hpf.

III. METHODOLOGY

1. Collection of eggs

Sexually mature Zebrafish (*Danio rerio*) were obtained from a local aquarium fish shop. The fishes were kept in a glass aquarium and maintain the temperature at 26° C. The fish were fed with a commercially available artificial diet (artemia) twice daily. The collection of eggs was done by using a mesh wire net, the net was placed at the bottom of the aquarium before placing the fish.

2. Preparation of media for embryos

For the development of embryos, E3 medium is used which is taken from the Zebrafish book by Chirstiane Nüsslein-Volhard and Ralf Dahm.

5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄, 10⁻⁵ % Methylene Blue. E3 can be made up as a 60x stock (without Methylene Blue). The 1x medium keeps under non-sterile conditions at room temperature for over a week. For 1 liter of a 60x stock solution, mix 17.2 g NaCl, 0.76 g KCl, 2.9g CaCl₂.2H₂O, and 4.9 g MgSO₄.7H₂O. Store stock in a fridge. Dilute 16 ml of 60x stock in distilled H₂O (dH₂O) to make up 1 liter of E3 (1x), and add 3-6 ml of 0.01% Methylene Blue as a fungicide.

3. Preparation of cadmium chloride solution

Different concentration of cadmium chloride was used to test the toxicity in Zebrafish embryos and larvae. Concentration taken for the test are 0, 25, 50, 75, 100, 125 mg/500ml respectively. These concentrations were added in the distilled water and mixed properly in separate containers and labeled respectively according to the concentration.

4. Exposure of embryos to cadmium chloride solution

The eggs were rinsed several times with tap water. For the exposure, Petri dishes were used. E3 medium was pore in the Petri plates and in each Petri plate 20μl of cadmium chloride concentrations were added 25, 50, 75, 100,125 mg/500ml respectively. Then in each dish containing cadmium chloride add 10 fertilized eggs with the help of a dropper. Embryos were continuously exposed to cadmium chloride in embryo medium from sphere stage (6 hpf) to end of segmentation stage (24 hpf). Incubate the eggs at 28° C. The experiment is performed in triplicates. Observe the embryos in a duration of 2 hrs. Compare the observation with embryos in control one.

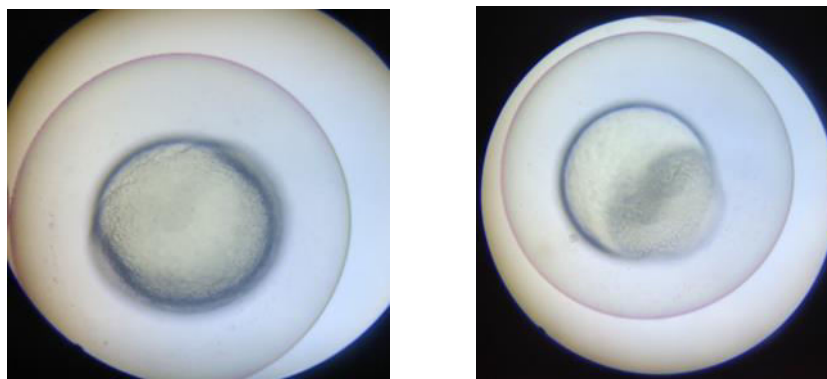


Figure 1: embryos used for the study

IV. RESULT

1. Exposure to cadmium during the early embryonic stage affect the formation of somites

Somite formation starts at the stage of 10 hpf as the concentration goes on increasing number of somites goes on decreasing. In concentrations 25, 50, 70 mg/500ml respectively the number of somites was 27 (22hpf) but in concentrations 100 and 125 mg/500 ml respectively the number was 25 (22hpf) compare to control one.

2. Exposure to cadmium during the early embryonic stage causes the irregular head formation

Zebrafish (*Danio rerio*) embryos exhibited head malformations in response to cadmium treatment. Boundaries of mid hindbrain become less prominent. The mid-hindbrain boundary which was prominent in control embryos was much less prominent in cadmium-treated embryos. In the concentration 25, 50 (mg/500ml) respectively shows normal head development but remaining concentration 75, 100; 125 (mg/500ml) respectively shows less prominent mid hindbrain region.

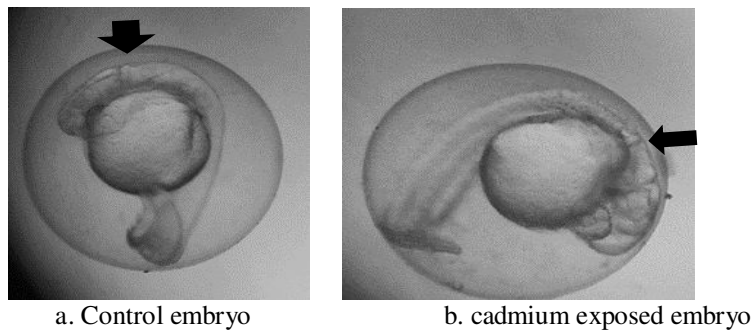


Figure 2: 28hpf embryos (a) control embryo with clear mid hindbrain neural line, (b) cadmium exposed embryos (100mg/500ml) with unclear mid hindbrain neural line.

3. Cadmium exposure can cause effects on hatching

The hatching period of the embryos is 48 hpf but when the embryos were treated with cadmium delay in the hatching was observed. Embryos in control media hatch at their particular time but embryos in concentrated media 75, 100, 125 mg/500ml hatched at the time period of 62 to 66 hpf.

4. Effects on fin formation when exposed in cadmium solutions

Development of the pectoral fin was very slow. Development of pectoral fin is to be done during the period of 42 hpf to 48 hpf (KIMMEL ET AL. 1995), here full development of the fin is seen at 66 hpf. In higher concentration, caudal fin development was seen reduced.

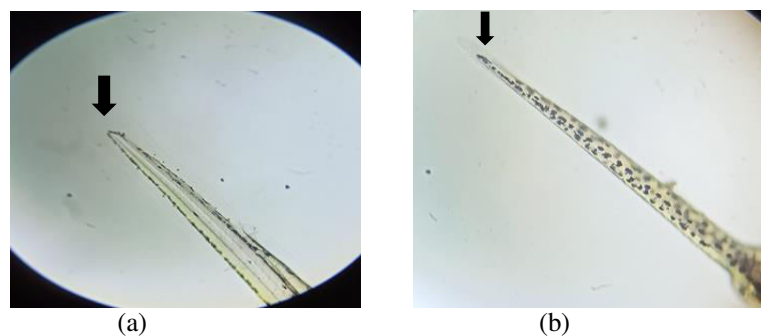


Figure 3: (a) 66hpf stage control larvae show normal caudal fin development, (b) cadmium exposed larvae shows reduced caudal fin formation.

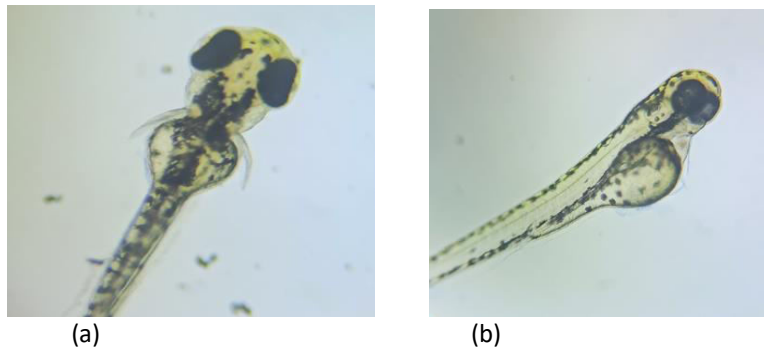


Figure 4: 66hpf larvae (a) control larvae show normal pectoral fin development, (b) cadmium-exposed larvae show reduced fin formation.

5. Effect of cadmium on eye development

Control embryos presented spherical eyes, consisting of the neural retina and the spherical lens enclosed, distinguished by a clear boundary but in cadmium-treated embryos develop a small eye. In cadmium-treated embryos, the neural retina covers more area and the lens becomes small in size.

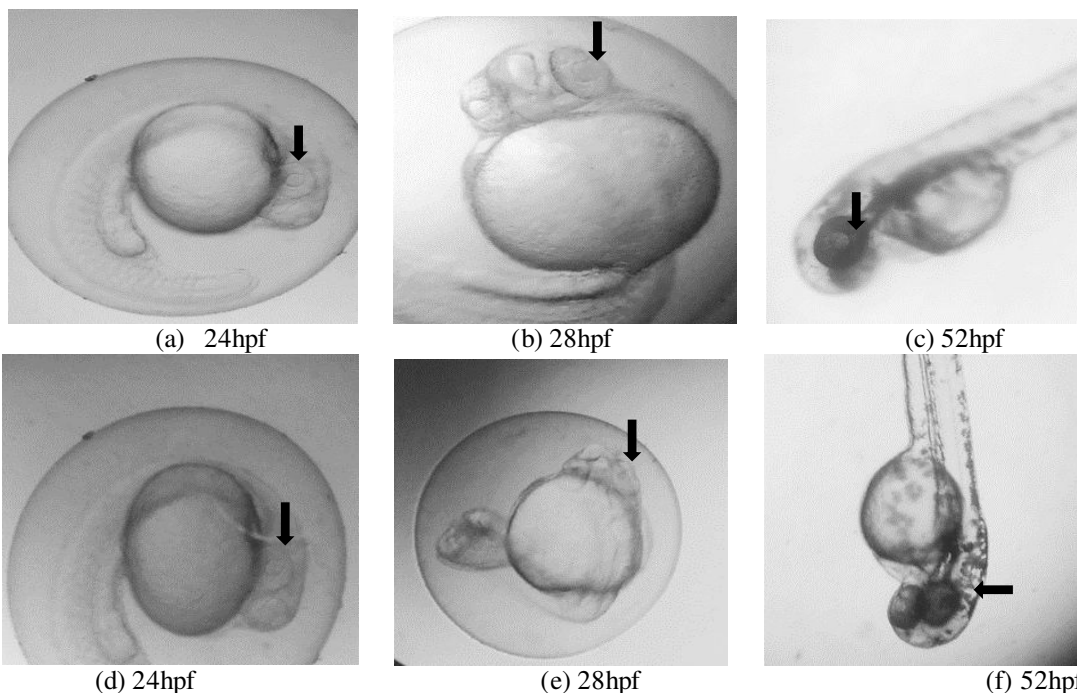


Figure 5: Effect of cadmium on eye development. (a), (b), (c) respectively shows control embryos with normal eye development, and (d) Lens of the eye is small in size, (e) Shape of the eye is not spherical, and (f) Lenses are absent.

I. Observation Table

Changes according to concentration						
Days / hours	Stage	25 mg/500ml	50mg / 500ml	75 mg/500ml	100 mg/500ml	125 mg/500ml
6hpf	Embryonic shield formed	No change as compared to control	No change as compared to control	No change as compared to control	No change as compared to control	No change as compared to control
16hpf	Segmentation period	14 somites	14 somites	14 somites	11 somites	10 somites
19hpf	Segmentation period	21 somites he eye lens is seen, Tail extended	21 somites The eye lens is seen, Tail extended	20 somites Eye lens is seen, Tail extended	20 somites Eye lens was not seen Tail extended	18 somites Eye lens was not seen, Tail is little bit blunt and small
22hpf	Segmentation period	27 somites neural lines are seen, eye lens becomes more prominent	27 somites neural lines are seen, eye lens becomes more prominent	27 somites Neural lines not seen	25 somites Eye lens are seen, Neural lines not seen	25 somites Eye lens are seen, Tail blunt, Neural lines not seen
25hpf	Pharyngula period	Heartbeat, fin fold is seen	Heartbeat, fin fold are seen	Heartbeat is seen	Heartbeat is seen, eye lens becomes more prominent	Heartbeat is seen, eye lens becomes more prominent
42hpf	Pharyngula period	Body pigmentation seen	Body pigmentation seen	Body pigmentation seen	Body pigmentation seen	Body pigmentation seen
46hpf	Hatching period	Few hatched	Hatching is not seen	Hatching is not seen	Hatching is not seen, eye development reduced	Hatching is not seen Eye development reduced.
48hpf	Hatching period	Some hatched, mid hindbrain regions become prominent	Few hatched, mid hindbrain region become prominent	Hatching is not seen, mid hindbrain region become prominent	Hatching is not seen, mid hindbrain region cannot be differentiated	Hatching is not seen, mid hindbrain region cannot be differentiated
66hpf	Larval stage	All embryos hatched, the pectoral fin is formed	All embryos hatched, the pectoral fin is formed	All embryos hatched, the pectoral fin is formed	All embryos hatched, pectoral fin does not detach from the yolk	All embryos hatched, pectoral fin does not detach from the yolk
69hpf	Larval stage	The caudal fin is observed and the yolk sac reduces	The caudal fin is observed and the yolk sac reduces	The caudal fin is observed and the yolk sac reduces	The caudal fin is seen but it was very reduced	Some larvae have reduced caudal fin but in some larvae, it was absent
72hpf	Larval stage	No change	No change	Movement becomes slow	Movement becomes slow	Movement becomes slow

Table 1: the table shows the changes on the morphology of the embryos and larvae of the Zebrafish when exposed in the different concentrations of cadmium.

V. CONCLUSION

Cadmium is one of those heavy metals which when present in the body of any organism like fishes (Zebrafish (*Danio rerio*)) can cause different types of developmental issues in embryos and larvae. If the concentration of cadmium is low in the medium then no effects are seen in the embryos and larvae but as the concentration increases different types of changes were observed the number of somites got decrease (24hpf), hatching of the embryos delayed by 24 hours means embryos should be hatched in the period of 48hpf, but the embryos which were exposed to cadmium hatched in the period of 66hpf, so it is clear that due to cadmium exposure delay in hatching is seen. The formation of the head neural lines which differentiate between the midbrain and hindbrain got mixed up during the developmental period. In the cadmium expose embryos the formation of the eye also got affected, the size of the eye become small and the inner spherical lens become small. Previous studies said that due to small eyes and reduce spherical lens the fish become blind. Cadmium also shows its effect on the pectoral and caudal fin formation, in the embryonic stage the development of the pectoral fin should get completed but here it is seen that the development of the fin was very slow. In the case of the caudal fin, some develop in a normal way but some seem to be reduced when compare with control medium larvae. The overall observation shows that if the fishes got expose to lower concentrations of cadmium no changes in the development and organs formation is seen, but if the concentration increases then the changes in the developmental stages are seen and the formation of reduced organs is also seen.

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