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***Research Article** (Open Access)*

ACUTE IMMUNOTOXICITTY OF XENOBIOTICS (1-CHLORO-2, 4-DINITROBENZENE) IN EMBRYONIC ZEBRAFISH

B. Dilshad Begum*, Mahathum Naseera M, Jothilekha S
P.G. & Research Department of Zoology, J.B.A.S. College for Women, Chennai, India
**Author for Correspondence: dilshadbegum.b@jbascollege.edu.in*

ABSTRACT
The present study was to elucidate the toxicity effect of a particular xenobiotic compound, 1-chloro-2,4-dinitrobenzene (DNCB), on embryonic zebrafish, as it is an emerging hazardous xenobiotic compound. The DNCB was taken at five different concentrations and induced in the embryonic zebrafish. The samples were observed at 24 hpf, 48 hpf, and 96 hpf. In each well, the zebrafish embryos had a count of 20. The toxicity assays were done in the embryonic and larval stages of zebrafish. In the embryonic stage, mortality rates and developmental defects were studied. The hatching rate was observed in the larval stage of the zebrafish. An increasing mortality rate and a decreasing hatching rate were observed. In each concentration, significant malformations and severe abnormalities were observed. As the concentration increases, the severity of the malformations also increases. The results of this study indicate the adverse effects of the 1-chloro-2,4-dinitrobenzene compound on embryonic zebrafish. The DNCB compound requires a relook for application in a variety of sectors.

Keywords: *Bioaccumulation, Xenobiotic Compound, LC-50, Embryo Toxicity*

INTRODUCTION
The xenobiotics are substances that are foreign to an organism's regular metabolism, and they are structurally complex. An emerging problem of the modern anthropogenic era is pollution of the environment with xenobiotics. The continual development of industry and the use of insufficiently balanced waste management result in increasing amounts of pollutants entering surface waters, particularly xenobiotic substances. These substances, such as dyes, pharmaceutical products, pesticides, and polycyclic aromatic hydrocarbons, have a negative impact on biodiversity. Their main routes of transfer to aquatic ecosystems include industrial and municipal wastewater discharges, as well as waste from households, hospitals and agriculture. The dinitrochlorobenzene (C6H3ClN2O4) is an essential component in the commercial synthesis of other chemicals. The 1-chloro-2,4-dinitrobenzene is an aromatic chemical that belongs to the family of organochlorines and nitro groups, which are particularly poisonous to aquatic creatures and can create long-term detrimental consequences in the environment. It is also considered a carcinogenic agent (PubChem Database, 2019). It is a powerful contact allergen that many dermatologists have employed in the past decade to treat benign skin conditions such as persistent viral warts1 and severe alopecia areata. Surprisingly, acute, single-dose, and single-substance exposure in adult populations is the main source of our current safety assessment and understanding of the toxicant's effect and method of action. The study's subject, the embryonic zebrafish, are a good substitute because of their rapid development, low maintenance requirements, resemblance to the function of the mammalian immune system, and high clutch size during the embryonic and larval stages, as well as their optical transparency, which makes it possible to see pathogens and lesions in real time (*Lesley R., Ramakrishnan L.* 2008). The embryonic zebrafish is a helpful model for studying the immune system. Their embryos have a number of defensive systems and are extremely sensitive to environmental parameters. Zebrafish are becoming increasingly popular in toxicological and immunology research due to their multiple benefits, including their small size, quick organogenesis, high fertility, minimal maintenance costs, and clear embryos.

Their genes and immune systems are quite similar to humans. Because of its high fertility, homology of genetic data to humans, and fast analysis times, the embryotoxicity model of zebrafish serves as an excellent model in toxicology research. This model is an appealing model for assessing the environmental risks of chemicals because they allow for small-scale, high-throughput investigations. The zebrafish, is employed in invitro research and developmental toxicity investigations as an embryonic and larval model.

MATERIALS AND METHODS

Characterization of Chemical

The toxicant that is used in this research was characterized by the Shimadzu 1800 UV-Vis Spectrophotometer (Japan). Once the characterization of the chemical was done by observing the UV peak in the graph, the research work proceeded with the next step. (Fig 1.1).

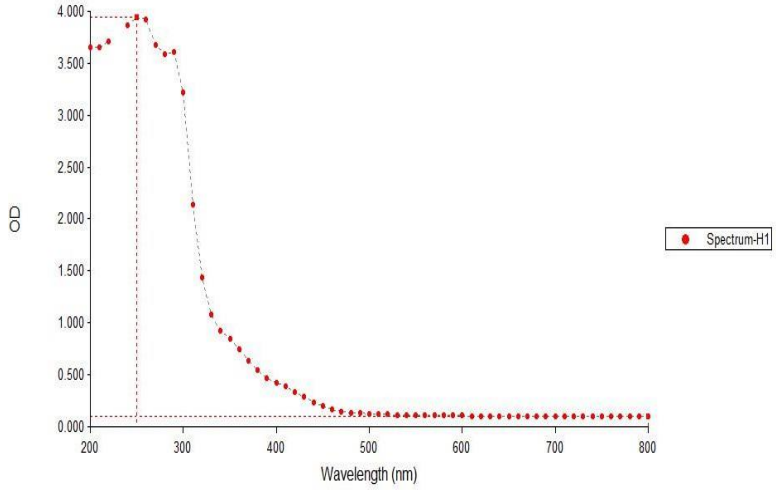


Fig. 1.1 - UV – Vis spectroscopy indicating the presence of DNCB

Sampling method

Fig. 1.2 The embryonic zebrafish *(Danio rerio)* - 5 hours post fertilization (hpf)

The embryonic stages of the zebrafish were collected from farms. The embryonic zebrafish (*Danio rerio*) were collected 5 hours post fertilization (hpf) (Fig. 1.2). They were acclimatized during the whole research process in the laboratory setup.

Preparation of Toxicant

The dinitrochlorobenzene crystals (Fig:1.3) were crushed and taken in the amount of 100mg. It was dissolved in 100 ml of distilled water using a hot magnetic stirrer for 45 minutes and cooled for 1 hour (Fig:1.4). The DNCB crystals were thoroughly dissolved in distilled water, and they were stored at room temperature (Fig:1.5).

Fig.1.3: Dinitrochlorobenzene Crystals

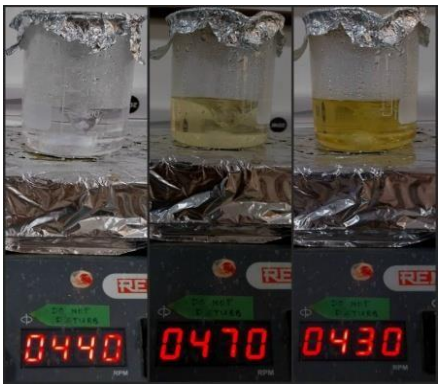


Fig.1.4: Dissolving dinitrochlorobenzene by using hot magnetic stirrer

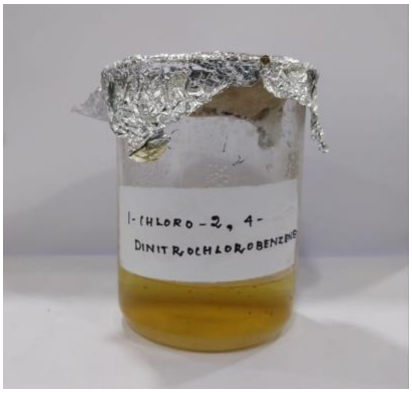


Fig. 1.5: Dissolved solution stored at room temperature

Sorting of the Embryos into the Well Plates

The embryonic zebrafish *(Danio rerio)* were collected 5 hour post fertilization (hpf). A Pasteur pipette was used to collect and remove all of the dead embryos (Fig:1.6). The embryos were cleansed with RO water.

Fig. 1.6: Pasteur pipette used to collect and remove all of the dead embryos.

and transferred to fresh petri plates. The sorted embryos were taken at a count of 20 in each well and introduced into the well plates. The RO water was poured into each well to acclimatize the embryos. All the experiments were carried out in triplicate, and the same number of embryos (n = 20) were followed throughout the whole study

Range Finding Test

The concentrations of the toxicant used in the experiment were determined by the LC-50 test. Several trials were carried out from a concentration of 1000 ul to 0 ul to find out the range of the concentration. The five final concentrations were determined as: 1.25 µl, 2.5 µl, 5 µl , 10 µl, and 20 µl.

Induction of the Toxicant

The well plates were divided into six columns, including the control (Fig:1.7). All the finalised concentrations of the toxicant were made into 1000ul by using distilled water. The embryos in the well plates were exposed to the respective five concentrations in triplicate (Fig:1.8).

Fig. 1.7: Toxicant introduced embryos

Fig. 1.8: Each concentration of toxicant was induced into the respective well plates using a micropipette

Observation of Toxicological Endpoints

The observation of toxicological endpoints was done at the embryonic and larval stages of the zebrafish. At 24 hpf, 48 hpf, and 96 hpf, the mortality and hatching rates were calculated in each well using five finalized concentrations. The defects in the development of the embryos at each concentration were studied using a stereo zoom microscope.

RESULT

In this present study conducted for to clearly show the detrimental impact of the particular xenobiotic compound, dinitrochlorobenzene on the embryonic zebrafish. The observation of toxicological endpoints using embryo toxicity assay. The mortality rate was calculated in each well with different concentrations, time points were used in the following experiments as shown in the Fig 2. The hatching rate was observed on third day and calculated as seen in Fig 3. The heartbeat per minute was observed on the second day post induction of the toxicant. In this study an increasing mortality rate and decreasing hatching rate were observed. And also the heart beat of the embryos were reduced in respect to concentration applied as in Fig 4. This study demonstrated that even the reduced utilization of 1- chloro 2- 4 -dinitrobenzene in embryonic zebrafish also causes of abnormalities and Malformations as detailed in Fig 5 in different concentration of Dinitrochlorobenzene were studied using a stereo zoom microscope.

Table 1: Concentration dependent change in the mortality rate during 24, 48 and 96hrs exposure of Dinitrochlorobenzene.

Group (µg)	No. of Zebrafish embryos	Mortality rate
Observation period: 24hrs		
Control	20	0
1.25	20	0
2.5	20	0
5	20	0
10	20	0
20	20	0
Observation period: 48hrs		
Control	20	1
1.25	20	1
2.5	20	4
5	20	6
10	20	20
20	20	20
Observation period: 96hrs		
Control	20	0
1.25	20	20
2.5	20	20
5	20	20
10	20	20
20	20	20

Figure 2: Concentration dependent change in the mortality rate during 24, 48 and 96hrs exposure of Dinitrochlorobenzene

Table 2: Concentration dependent change in the hatching rate during 24, 48, 96hrs exposure to Dinitrochlorobenzene.

Group (µg)	No. of Zebrafish embryos	Hatching rate in percentage
Observation period: 24hrs		
Control	20	0
1.25	20	0
2.5	20	0
5	20	0
10	20	0
20	20	0
Observation period: 48hrs		
Control	20	0
1.25	20	0
2.5	20	0
5	20	0
10	20	0
20	20	0
Observation period: 96hrs		
Control	20	100
1.25	20	60
2.5	20	50
5	20	0
10	20	0
20	20	0

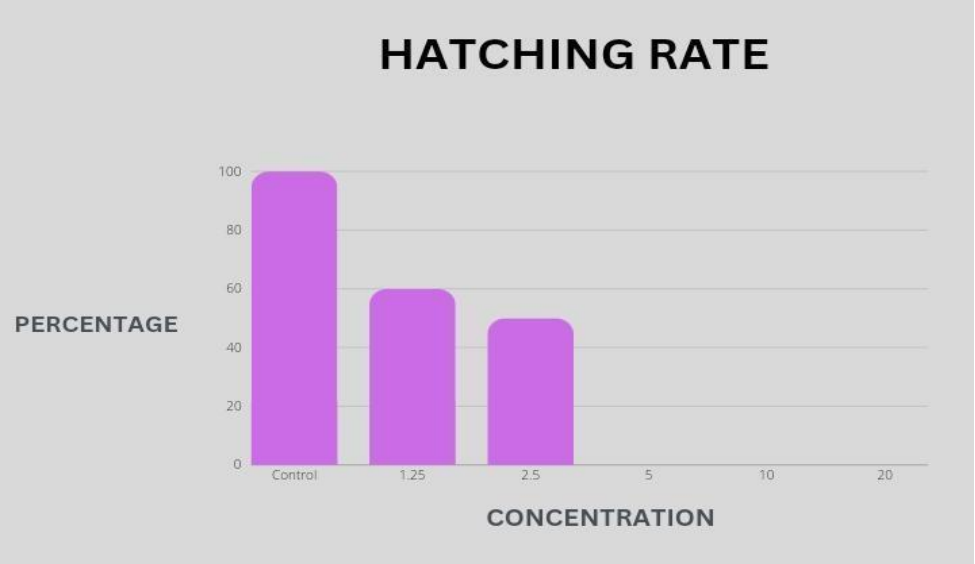


Figure 3: Concentration dependent change in the hatching rate during 24, 48, 96hrs exposure to Dinitrochlorobenzene

Table 3: Concentration dependent change in the heartbeat per Minute during 24, 48, 96hrs exposure to Dinitrochlorobenzene.

Group (µg)	No. of Zebrafish embryos	Heartbeat per minute
Observation period: 24hrs		
Control	20	0
1.25	20	0
2.5	20	0
5	20	0
10	20	0
20	20	0
Observation period: 48hrs		
Control	20	64
1.25	20	60
2.5	20	56
5	20	44
10	20	36
20	20	24
Observation period: 96hrs		
Control	20	0
1.25	20	0
2.5	20	0
5	20	0
10	20	0
20	20	0

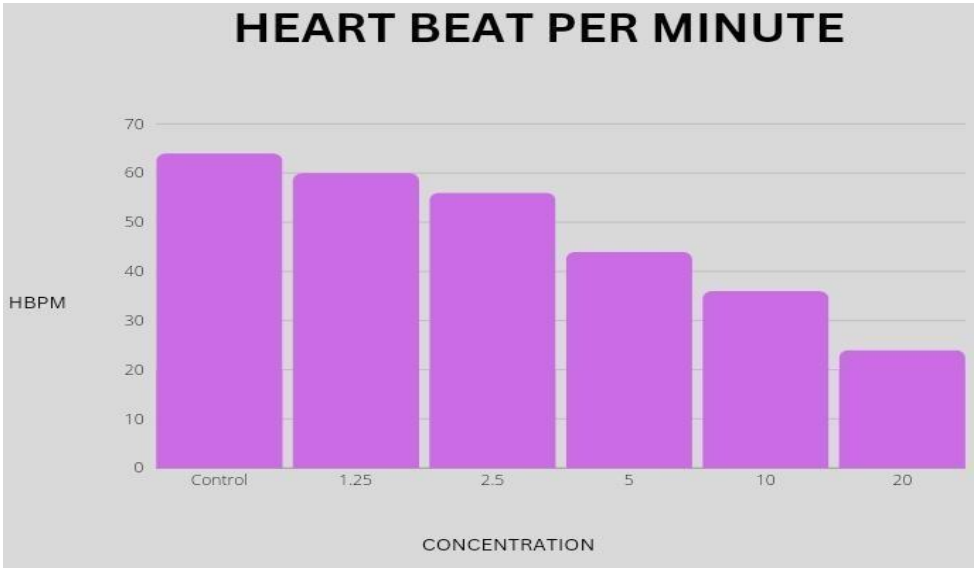
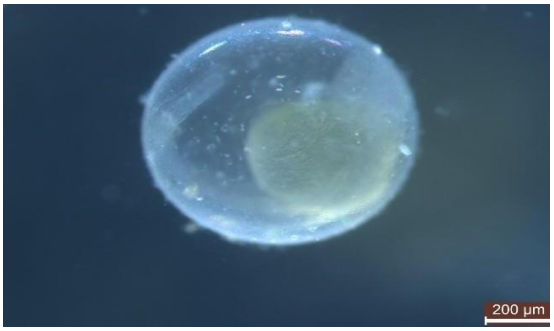


Figure 4: Concentration dependent change in the heartbeat per Minute during 24, 48, 96hrs exposure to Dinitrochlorobenzene

Control
No developmental defects were found



1.25 µl
Malformation of the head and there is no development of eyes were shown

2.5 µl
Yolk sac was irregular in shape and around the head. Tail was not fully developed were observed

5 µl
The formation of teratoma region was found and Tail was bended.

10 µl - Yolk sac was abnormal and stunted growth of the tail were observed in the 10µl

20 µl
The absence of head, Yolk sac was irregular in shape and there were no formation of tail seen in 20 µl concentration. And also the chorion was found to be crystallized

Figure 5: **Developmental defects of the embryos in each concentration were studied using a stereo zoom microscope.**

Developmental defects of the larval Zebrafish

The larval stage of the zebrafish was stained with eosin, which showed notable abnormalities in the anterior region (Fig. 6.1). The eyes of the larval zebrafish were significantly enlarged. The larval zebrafish also showed a malformed body in the leishman stain (Fig. 6.2).

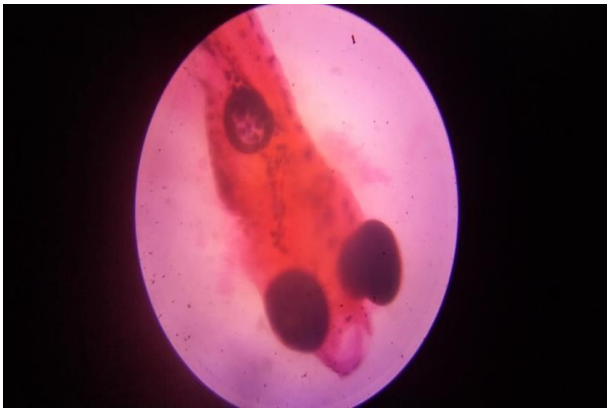


Fig. 6.1: The eyes of the larval zebrafish were significantly enlarged

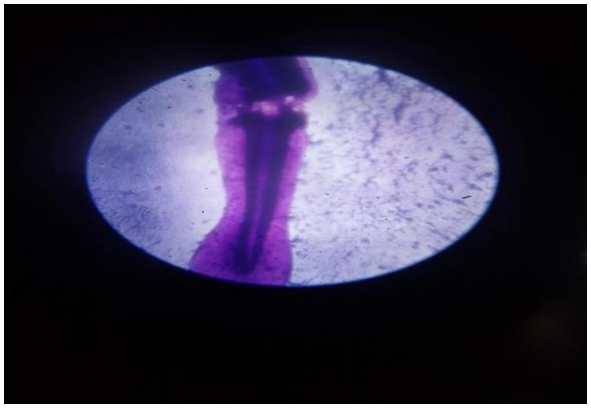


Fig. 6.2: The larval zebrafish showed a malformed body in the leishman's stain

DISCUSSION

1-chloro-2,4-dinitrobenzene (DNCB) is a powerful contact allergen that has been used to treat a variety of dermatological disorders over the last decade, including benign skin diseases and alopecia areata (Andrew N. Lin, 2013). Some HIV-positive people have applied a very dilute solution to a tiny area of skin to boost their immune system performance. This chemical can accumulate over time and is particularly harmful to aquatic creatures. Topical immunotherapy with contact sensitizers is a successful and well recognized treatment option for chronic alopecia areata (Mohan K H et al., 2008). 1-chloro-2,4-dinitrobenzene has been utilised in HIV treatment trials (Paul Urso et al., 2008). During over a decade of topical DNCB worldwide, the only recorded adverse effect has been localized dermatitis at the application site (KH Mohan et al., 2008). This substance stimulates the immune system, which helps remove warts. Once the cells have been taught to distinguish DNCB as a foreign molecule, the immune system will attack the warts. DNCB caused minor allergic contact dermatitis and slight transitory hyperpigmentation at the location of application.

The early life stages of zebrafish *(Danio rerio)* provide a diverse model system for studying the efficacy and safety of medications or other substances on human and environmental health (Alena Tierbach et al., 2020). Body changes in zebrafish larvae indicate an immediate inflammatory response, which may indicate a similar immunotoxicity mechanism for the majority of environmental contaminants (Davide Di Paola, et al., 2021). Beyond their use in acute toxicity testing, they are ideal models for studies aiming at understanding toxic processes and predicting potential detrimental and long-term consequences. Because of the zebrafish's transparency in its early stages, embryo growth is easy to observe and examine. The reduced hatching rate was caused by structural and functional abnormalities during embryonic development since hatching is a crucial stage of zebrafish embryogenesis. (Liu L., et al., 2016). Zebrafish embryos can absorb substances that have been put to the water. The zebrafish is perhaps one of the most widely used model organisms for genetic and developmental research in all of its stages. The zebrafish has become known for its remarkable regenerative ability and quick embryonic growth.

CONCLUSION

The toxicity analysis was carried out to study the effects of dinitrochlorobenzene in aquatic ecosystems, as this ecosystem is a reservoir of all industrial, pharmaceutical, and food wastes. All the xenobiotic compounds will accumulate in this ecosystem, and they are potentially hazardous to the fish population. To analyse the effects of DNCB occurring in embryos and larvae, different concentrations of diluted dinitrochlorobenzene were used Under OECD Guidelines, 236, FET Test. The embryos were exposed to toxicants and studied for three days. An increasing mortality rate and decreasing hatching rate was observed. The significant abnormality, which is the delayed hatching rate, was observed in the larval stage, which was on the third and fourth days. The heartbeat of the embryos was reduced with respect to the concentration applied, and at the higher concentration of 20 ul, the heartbeat declined and the mortality of the embryos was observed. The stereozoom pictures indicated the main abnormalities of the chorion, yolk sac, and body in response to the rising concentration of DNCB. Thus, this study effectively illustrates the harmful effects of the particular xenobiotic component dinitrochlorobenzene on embryonic zebrafish.

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