Journal of Advanced Zoology
ISSN: 0253-7214
Volume 45 Issue 4 Year 2024 Page 96-105

Teratogenic Effects of Di(2-ethylhexyl) phthalate (DEHP) on Zebrafish Embryos

Arundhati Dahiwal[1], Roshan C. D'Souza[1]*

[1]*Department of Zoology, Sophia College for Women (Auto.), Mumbai 400 026, M.S., India*

***Corresponding Author:** Roshan C. D'Souza*
E-mail: roshan.dsouza@sophiacollege.edu.in

Abstract

Developmental toxicity studies help to understand the impact of a pollutant on this crucial phase of the living organisms which can affect their population dynamics. Zebrafish has become an ideal model for studying environmental and embryo toxicity. The present study was carried out using zebrafish embryos for assessing the environmental toxicity of Di(2-ethylhexyl) phthalate (DEHP) which is universally considered to be an omnipresent environmental contaminant as it is the most widely used plasticizer. The embryos were exposed to DEHP for a range of five concentrations of 0.2, 20, 80, 140 and 200 µg/L for the duration of 96 hours. The treatment resulted in increased mortality and decreased hatch rate, hatchability and heartrate. It also induced teratogenic endpoints like yolk sac edema, pericardial edema and spinal deformity in the embryos which increased in dose and time dependent manner.

Keywords – Di(2-ethylhexyl) phthalate (DEHP), Zebrafish embryos, Developmental toxicity, Edema

Introduction

Phthalates are widely produced and used in a wide range of products such as plastic, vinyl, personal care products, children's toys, and "fragrance". Phthalates can accumulate in adipose tissues after being easily absorbed in the bloodstream[1]. The spread of phthalates in the environment has sparked public concern due to their capacity to cause liver cancer, structural and developmental defects, and lower sperm count in males[2]. Male and female reproductive systems are more vulnerable to phthalates. Human milk contains a larger concentration of hydrophobic phthalates[3]. As a result, maternal exposure to phthalates is becoming increasingly concerning for human foetuses and babies. Because of major concern around pediatric exposure recent research has received considerable attention to one of the globally used phthalate, Di(2-ethylhexyl) phthalate (DEHP). Like all the phthalates this phthalate is ubiquitous contaminants in air, food, water bodies, soil and sediments[4-5]. In 2008, it was reported[5] that DEHP was produced at an annual rate touching more than 2 million tons across the globe which makes it evident that DEHP has been in use consistently due to its plasticizing properties and low cost. It is universally considered to be an omnipresent environmental contaminant because it is the most widely used plasticizer. It is mainly used in PVC formulation for its wide range of applications including medical devices, cosmetics, personal care products, clothing, furniture and other home appliances, car products, etc.[6].

Since DEHP can easily find its way into the environment, it can readily enter the body through inhalation, ingestion and dermal contact. Food may also contain DEHP. According to epidemiological studies carried out

in different organisms it is found that DEHP can be found in meat and lipid-rich products such as fats and dairy products at higher concentrations[5,7]. The EU designated DEHP as a substance of very high concern (SVHC) under Article 59(10) of the REACH Regulation (Registration, Evaluation, Authorisation and Restriction of Chemicals) (EC) No 1907/2006[8]. Studies have reported that DEHP is up to 0.02–264 mg/kg in agricultural soil, is 0.01–30.1 mg/kg in urban soil, is 3640 ng/m^3 in the air, is 634.2 µg/g1394.7 µg/g in indoor dust, is 0.55–8351.85 µg/L in drinking water[9-10], and is 2.6 µg/L in waste water treatment plants from India[11]. This has therefore raised some concerns about its safety and potential effects on human health.

Zebrafish has become an ideal model for studying environmental and embryo toxicity because of their experimental characteristics, which include small size, development outside of the mother, rapid embryonic development, optical transparency, easy and cheap maintenance of both embryos and adults, and a high degree of genomic homology to mammals[12-13]. The easy monitoring of morphological development and the evaluation of possible malformations while identifying toxicity endpoints are possible due to the transparency of the embryos[14].

The study was carried out for acute exposure of 96 hours to varying concentrations of DEHP (0.2, 20, 80, 140 and 200 µg/L) on zebrafish embryos as developmental toxicity studies help to understand the impact of pollutant on the crucial developmental phase of an organism. The results will provide better understanding of ecotoxicity of DEHP and appropriately assess the threat that phthalates can cause to the environment.

Materials and Method

Test solution

Di(2-ethylhexyl) phthalate (DEHP) was purchased form Sigma-Aldrich, USA, Product No. 47994. The exposure concentrations of DEHP of 0.2, 20, 80, 140 and 200 µg/L were prepared with the embryo medium.

Model animal maintenance, breeding and collection of eggs

Eggs were collected from the zebrafish maintained in the CCSEA registered zebrafish maintenance and breeding facility (Registration No.1936/PO/Re/S/17/CPCSEA) under standard laboratory conditions, i.e. 28°C ± 1°C and photoperiod of 14:10 hours light:dark. The fish were fed twice a day with readymade dry fish food and once a day with *Artemia*. The pairs were kept in the breeding chamber with mesh at the bottom to prevent cannibalization of the eggs. The eggs were carefully collected and rinsed thoroughly with tank water to get rid of dirt/waste particles. They were then transferred to a petri plate containing freshly prepared embryo (E3) medium. After one hpf (hour post-fertilization) the eggs were screened under the stereo microscope to select healthy and fertilized eggs, and to discard the unfertilized ones.

Experimental setup and embryo toxicity studies

The study was carried out according to the guidelines of OECD Test No. 236: Fish Embryo Acute Toxicity (FET) Test[15]. Six 24-well plates were used, one for control and five containing respective concentrations of DEHP (0.2, 20, 80, 140 and 200µg/L). Sets in triplicate were used. The fertilized eggs at 2 hpf were gently transferred into the plates, one egg in each well. Control well plates were filled with 2 ml of E3 medium. Each of five plates of the treatment groups had 20 embryos in DEHP solution and 4 embryos as an internal control. The embryos were incubated at 28°C ± 1°C and observed every 24 hours under stereo microscope upto 96 hpf. The embryos were screened daily and scored for survival, mortality, hatching, heart rate, developmental abnormalities, morphological malformations like yolk sac edema, pericardial edema, bent spine, and compared with control group. LC_{50} was calculated using probit analysis.

Statistical analysis

Results were expressed as Mean ± SD. Statistical analysis was done using One-way ANOVA followed by Bonferroni test for post hoc analysis. Differences at $P < 0.05$ were considered significant. LC_{50} was calculated using Finney's Probit data analysis method.

Results and Discussion

The embryos from the control group and internal control were healthy and showed normal growth throughout the observation period of 96 hours. The embryos hatched normally at 48 hpf (Fig 8A).

LC_{50} value

In the present study LC_{50} was observed to be 225.16 µg/L at the end of 96 hours (Table 1). The lethal concentration that caused 50% mortality (LC_{50}) of DEHP was found to be 2.5 µg/L in zebrafish embryos at 72 hpf[16]. LC_{50} value of DEHP for zebrafish embryos from 72 to 168 hpf was reported as 54.02 mg/L[17].

Table 1: LC_{50} of zebrafish embryos exposed to different concentrations of DEHP (µg/L) at 24, 48, 72 and 96 hpf using probit analysis

Exposure Period (Hours)	LC50 (µg/L)
24	709.1
48	614.69
72	461.37
96	225.16

Cumulative mortality

The cumulative mortality was calculated for 24, 48, 72 and 96 hpf. The cumulative mortality was found to be significantly increased in a time and dose dependent manner (Fig 1). The results of present research are in agreement with the earlier work done where DEHP exposed embryos showed increase in the mortality rate[18-20]. Mortality rate was also found to be increased in MEHP treated zebrafish embryos[21].

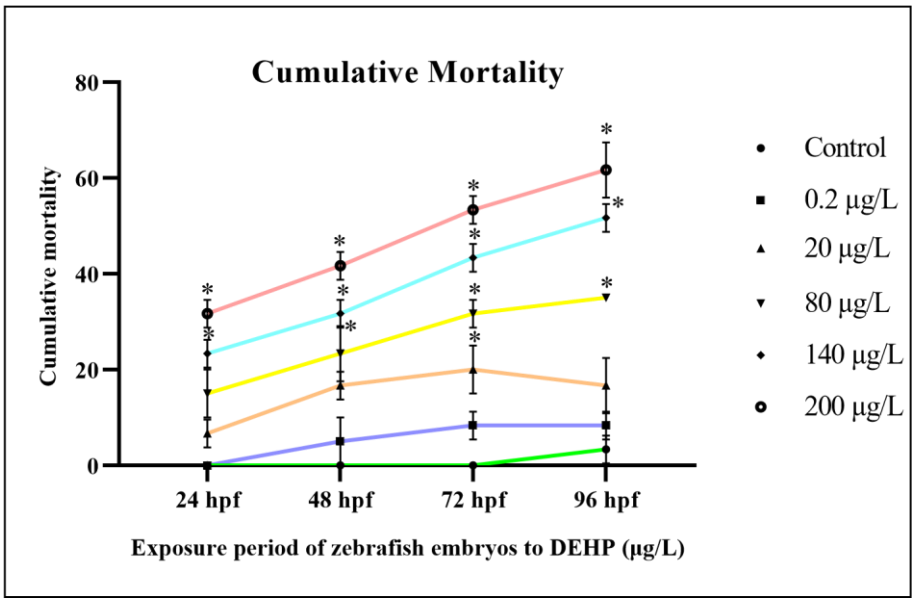

Fig 1: Cumulative mortality of zebrafish embryos exposed to different concentrations of DEHP (µg/L) at 24, 48, 72 and 96 hpf. * Denotes significant differences between control and treatments at different time periods at P < 0.05

Hatch rate and Hatchability

Hatching is known to be an important event in the life cycle of a fish. It is a crucial period of embryogenesis and hence a key point in the biological clock of fish. The most widely used endpoint in developmental toxicity studies of fish is hatching time or rate of hatching[22]. Hatching is the result of combined action of hatching enzyme (chorionase), secreted by the hatching gland cells of the embryo, increased perivitelline pressure and muscular contractions[23]. The hatch rate is the ratio of number of eggs hatched at time "X" to the number of eggs or embryos alive at time "X", whereas, hatchability is the ratio of number of eggs hatched with respect to the total number of fertile eggs. Hatch rate was calculated from 50 hpf to 65 hpf at interval of 5 hours. It was observed that hatching was delayed with increasing DEHP concentration (Fig 8E). As compared to control, there was significant difference in hatch rate of embryos exposed to 80, 140 and 200 µg/L of DEHP at 65 hpf (Fig 2). Significant decrease in hatchability was observed in 80, 140 and 200 µg/L of DEHP exposed embryos (Fig 3). Significant delay in hatching time was found in embryos of Japanese medaka exposed to 0.1, and 1.0 µg/L of DEHP[24].

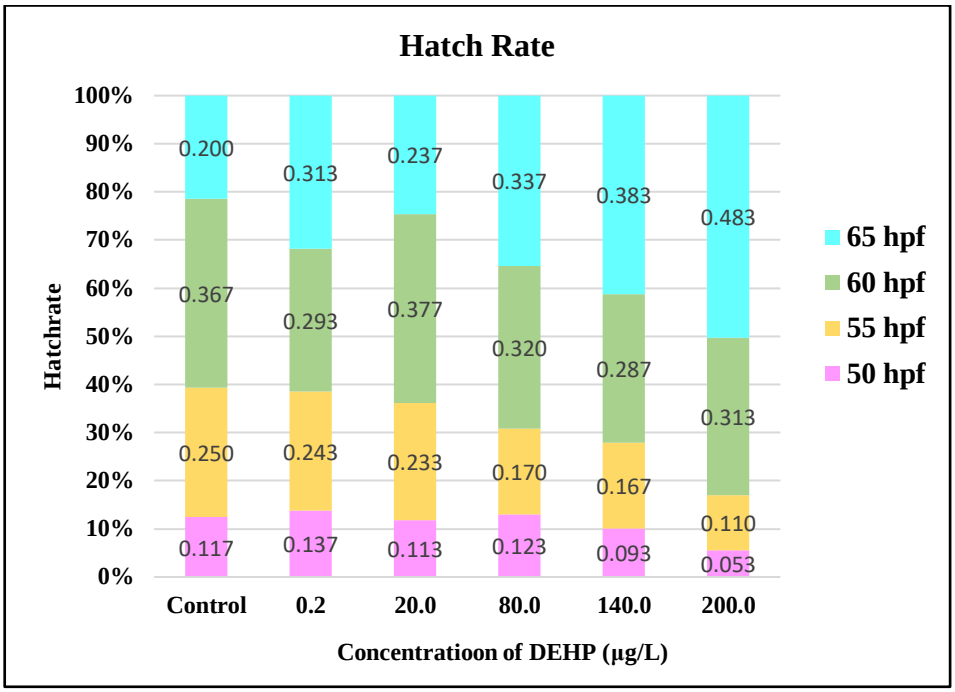

Fig 2: Hatch rate of zebrafish embryos exposed to different concentrations of DEHP (µg/L) from 50 to 65 hpf expressed in %. Data is expressed as Mean ± SD.

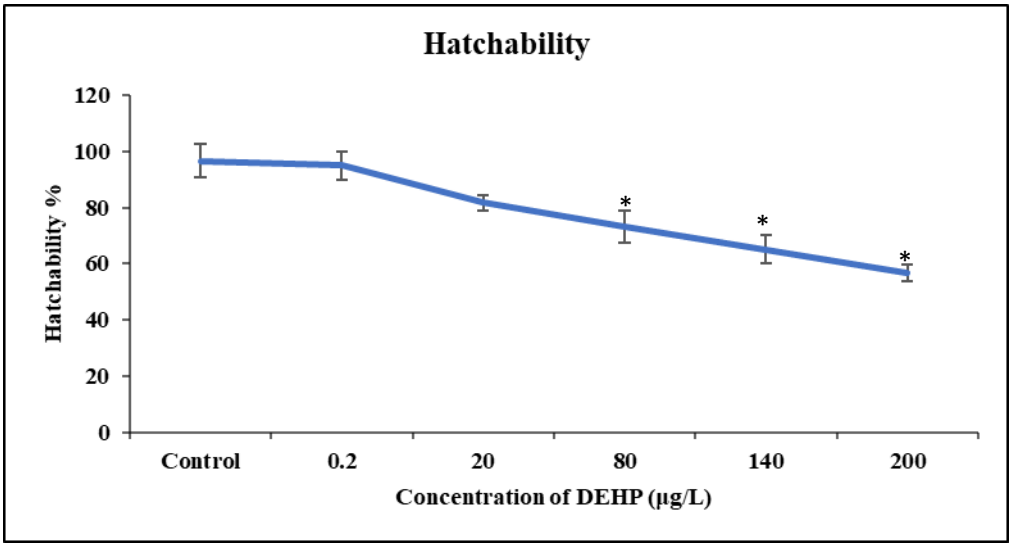

Fig 3: Hatchability of zebrafish embryos exposed to different concentrations of DEHP (µg/L) after 96 hpf. * Denotes significant differences between control and treatments at different time periods at P < 0.05

Pericardial edema and Heart rate

The most sensitive teratogenic effect caused by DEHP was pericardial edema (Fig 4). Pericardial edema was observed in DEHP treated embryos at 48, 72 and 96 hpf (Fig 8B, 8C, 8E). Embryos treated with 0.2 and 20 µg/L of DEHP showed pericardial edema at levels similar to control group. The embryos exposed to 80 µg/L of DEHP showed significant increase in pericardial edema from 72 hpf while 140 and 200 µg/L of DEHP showed significant difference from 48 hpf onwards. Zebrafish embryos exposed to DBP showed severe deformities including pericardial edema, delayed yolk sac absorption, bent notochord, small eyes, short body length[25]. Reduction in heart rate of DEHP exposed zebrafish embryos was observed in the study. Embryos exposed to 0.2 and 20 showed no significant change while 80 µg/L showed significant decrease from 72 hpf. 140 and 200 µg/L of DEHP showed significant decrease from 48 hpf onwards (Fig 5). Zebrafish embryos exposed to 250 µg/L of DBP and DEHP showed reduction in heart rate along with pericardial edema[26]. Zebrafish are highly useful, appropriate and suitable model for the study of cardiovascular development and cardiotoxicity[27-28]. The results of present study suggests that DEHP impairs the cardiac structure and function.

One possible explanation for edema could be a disturbance of embryonic osmoregulation[29]. The heart might be a priority or main target for DEHP toxicity during the crucial development period of zebrafish embryos.

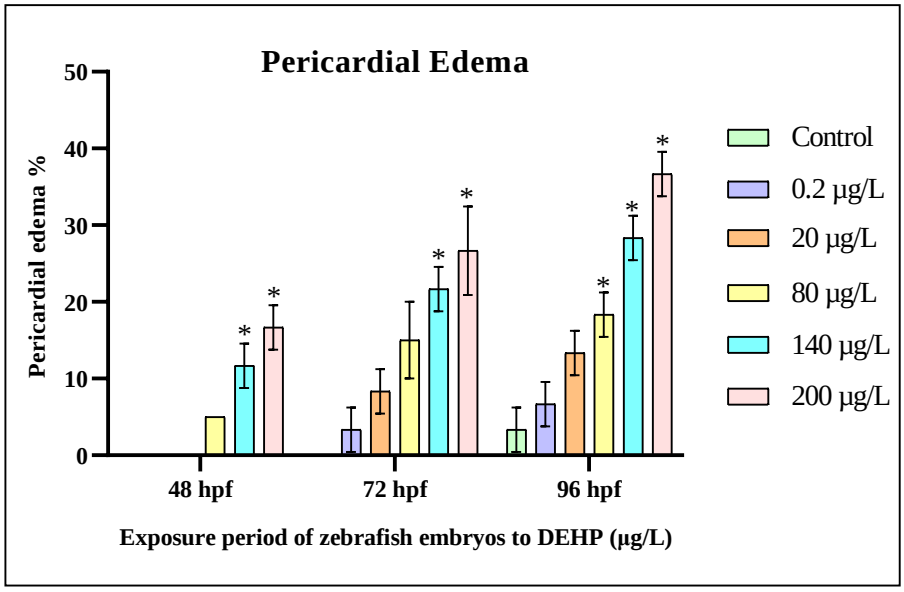

Fig 4: Pericardial edema occurring in zebrafish embryos exposed to different concentrations of DEHP (µg/L) at 48, 72 and 96 hpf. * Denotes significant differences between control and treatments at different time periods at P < 0.05

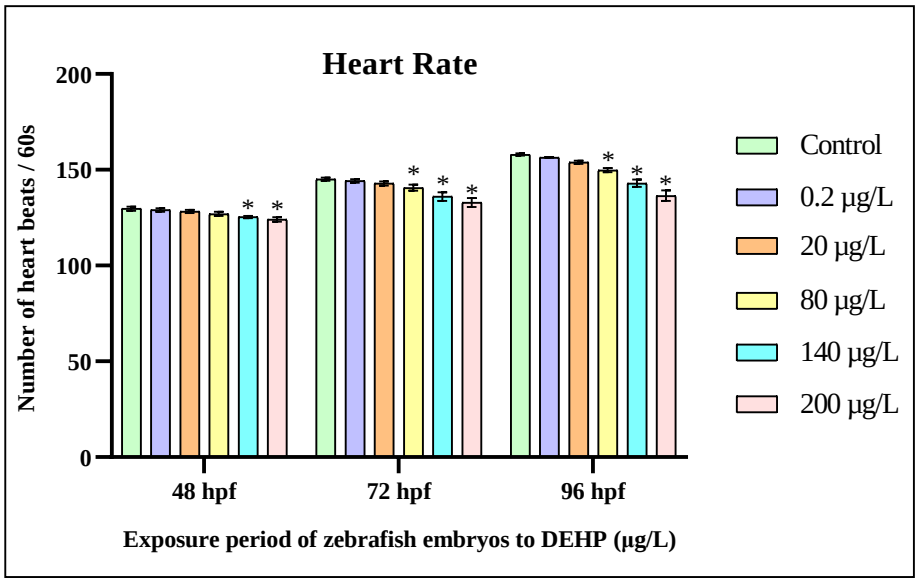

Fig 5: Number of heart beats occurring in zebrafish embryos exposed to different concentrations of DEHP (µg/L) at 48, 72, and 96 hpf for 60s. * Denotes significant differences between control and treatments at different time periods at P < 0.05

Yolk Sac Edema

The first extra-germinal organ to form during gastrulation in fish is the yolk sac, which aids in the future development of embryo[30]. Hence, another important morphological deformity seen in DEHP treated embryos was yolk sac edema (Fig. 8B, 8C, 8D, 8E, 8F). There was no significant effect on the embryos exposed to 0.2 µg/L of DEHP. 20 µg/L exposed embryos showed significant increase at 96 hpf while 80 µg/L showed the difference from 72 hpf onwards. Significant increase in edema was observed in 140 and 200 µg/l of DEHP treated embryos from 48 hpf onwards (Fig 6). In developmental toxicity studies of zebrafish yolk sac edema is very commonly observed pathology and a sensitive toxicological outcome for evaluation of embryos[31]. Yolk sac edema could also result into cardiac defects. In oviparous fish, embryos use endogenous yolk nutrients

present or buildup in the oocyte during the development of embryos[32]. Nutrient supply might get blocked by the impairment in the yolk sac of the developing embryos and thus the energy limitation could affect the heart function[33]. This is also a potential reason for pericardial edema[34]. A pathology known as 'blue sac syndrome' is characterized by yolk sac edema and pericardial edema[35].

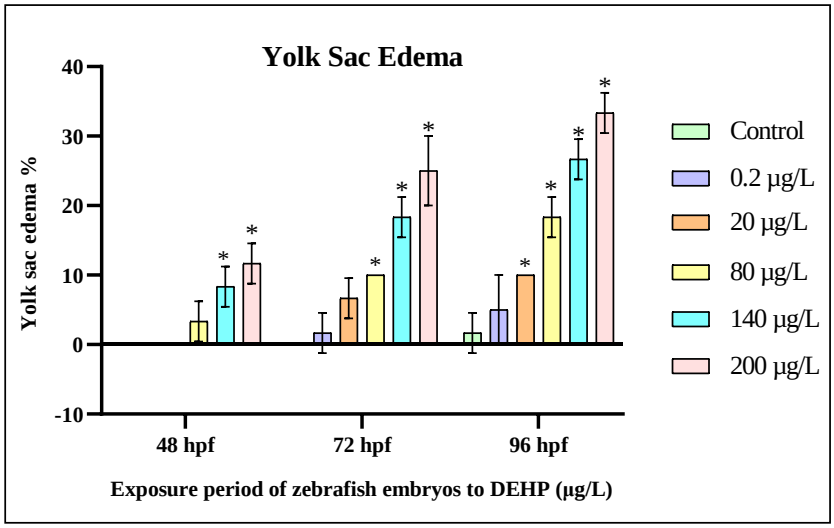

Fig 6: Yolk sac edema occurring in zebrafish embryos exposed to different concentrations of DEHP (µg/L) at 48, 72, and 96 hpf. * Denotes significant differences between control and treatments at different time periods at P < 0.05

Spinal Deformities

Spinal deformities like scoliosis, lordosis and kyphosis were found in zebrafish embryos exposed to DEHP (Fig 8B, 8C, 8D, 8E, 8F). 140 and 200 µg/L of DEHP showed significant difference in spinal deformities from 72 hpf onwards (Fig 7). Zebrafish and humans share a high degree of genetic conservation and have similar structure and morphology of the spine[3639]. Hence there are various benefits of using zebrafish as model for studying human spine deformities. Among several malformations, spinal curvature is another malformation often seen in embryos and larvae of zebrafish exposed to toxic chemicals[40]. The three varied spine deformities may result from a variety of conditions, such as differential toxicant buildup, impaired neuromuscular coordination and decreased AChE activity[40]. Changing amino acid composition and decreased amounts of collagen in the spinal column can also result into spinal deformities[41]. DEHP may get accumulated in the embryos and interfere with neuromuscular activity. This could have adversely led to spinal curvature in DEHP exposed embryos.

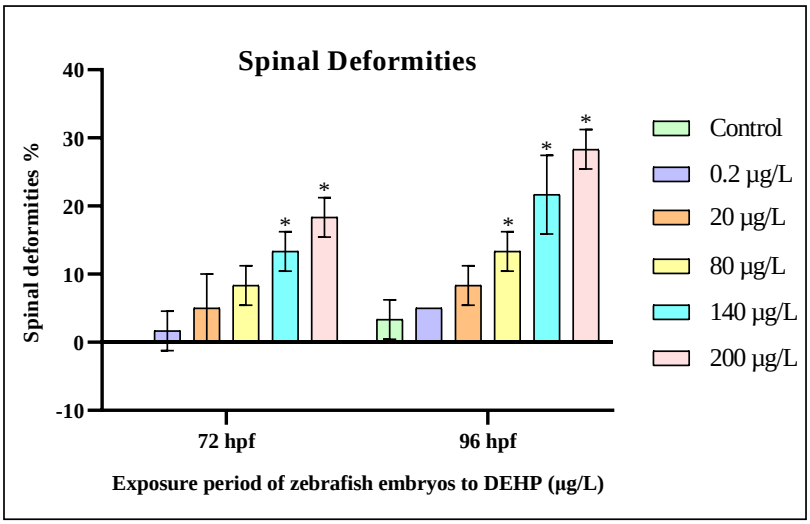

Fig 7: Abnormal curvature of the spine occurring in zebrafish embryos exposed to different concentrations of DEHP (µg/L) at 72 and 96 hpf. * Denotes significant differences between control and treatments at different time periods at P < 0.05

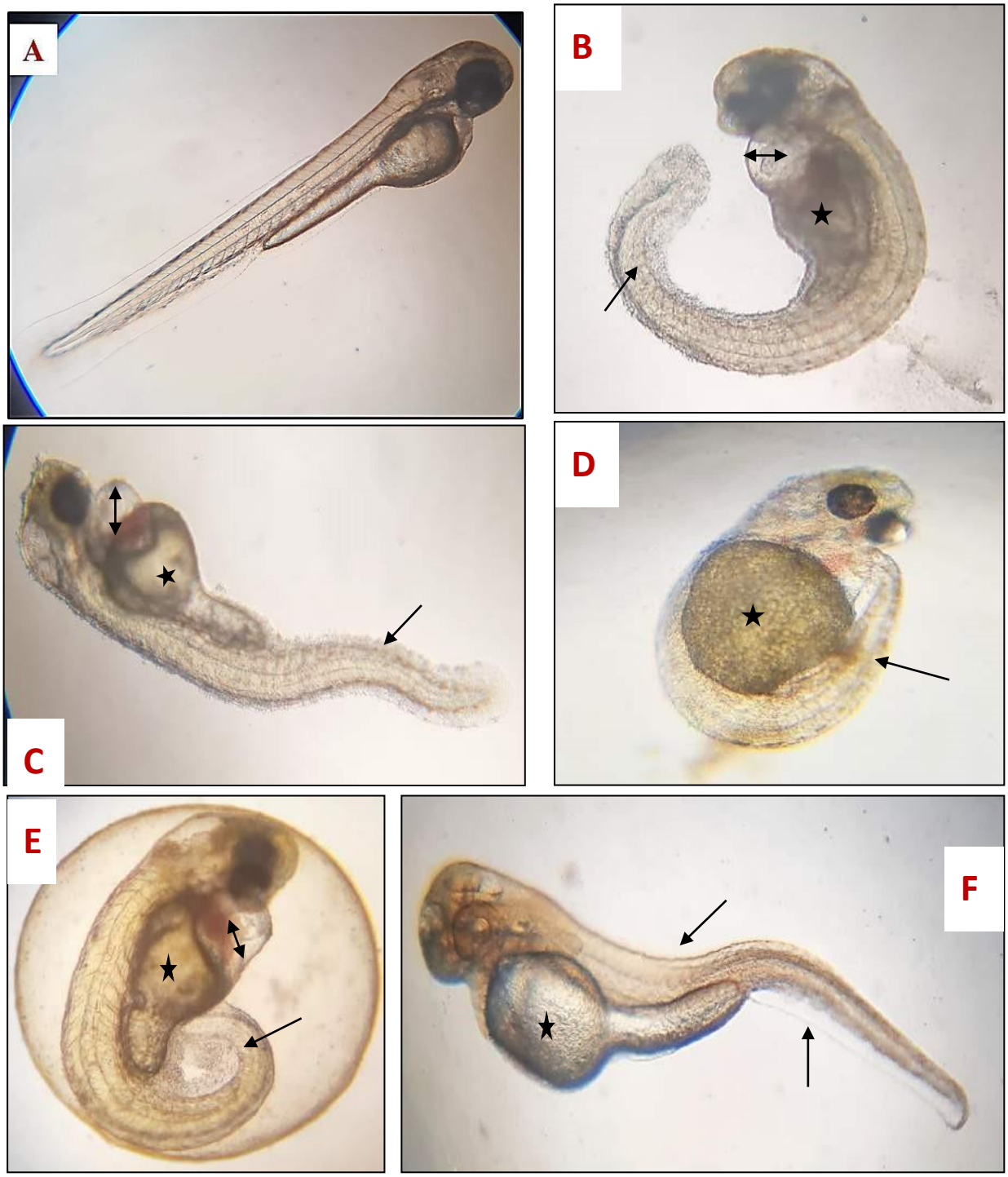

Fig 8: Morphological effects of DEHP on zebrafish developmental stages. A - Normal embryo at 48 hpf; B, C and F - embryo with pericardial edema, yolk sac edema and spinal deformity at 48 and 96 hpf respectively; D - embryo with pericardial edema and spinal deformity at 72 hpf; E – embryo with pericardial edema, yolk sac edema, spinal deformity and delayed hatching at 72 hpf. Star denotes pericardial edema, Single side arrow denotes spinal deformity and double side arrow denotes yolk sac edema.

Conclusion

The results of present study indicate that DEHP can cause developmental abnormalities and toxicity in the embryos of zebrafish. The mortality and abnormalities increased in concentration and time dependent manner. Thus, it was ascertained that DEHP cause harmful effects on developmental stages, thereby disturbing the population dynamics in the given aquatic ecosystem contaminated with DEHP. Hence, this study calls for a

more judicious use of DEHP and its possible replacement with other substitute compounds as plasticizers in order to mitigate its aquatic environmental toxicity.

Acknowledgement

The authors would like to acknowledge the infrastructural support given by the Suman Tulsiani Research Centre and the CCSEA registered zebrafish maintenance facility of Sophia College for Women (Autonomous), Mumbai.

References

1. Walsh Emma (2019). Date with Plastic: Plasticizers in our Cosmetics. *Medium.*
2. Bauer, M. J., & Herrmann, R. (1997). Estimation of the environmental contamination by phthalic acid esters leaching from household wastes. *Science of the Total Environment*, *208*(1-2), 49-57.
3. Frederiksen, H., Skakkebaek, N. E., & Andersson, A. M. (2007). Metabolism of phthalates in humans. *Molecular nutrition & food research*, *51*(7), 899-911.
4. Li, F., Liu, Y., Wang, D., Zhang, C., Yang, Z., Lu, S., & Wang, Y. (2018). Biodegradation of di-(2-ethylhexyl) phthalate by a halotolerant consortium LF. *PLoS One*, *13*(10), e0204324.
5. Rowdhwal, S. S. S. & Chen, J. (2018). Toxic Effects of Di-2-ethylhexyl Phthalate: An Overview. *BioMed research international*, *2018*, 1750368.
6. Koo, H. J., & Lee, B. M. (2004). Estimated exposure to phthalates in cosmetics and risk assessment. *Journal of Toxicology and Environmental Health, Part A*, *67*(23-24), 19011914.
7. Serrano, S. E., Braun, J., Trasande, L., Dills, R., & Sathyanarayana, S. (2014). Phthalates and diet: a review of the food monitoring and epidemiology data. *Environmental health : a global access science source*, *13*(1), 43.
8. European Union. Article 59(10) of the REACH Regulation. (EC) No 1907/2006. https://echa.europa.eu/substance-information/-/substanceinfo/100.003.829
9. Li, Y., Xie, D., He, L., Zhao, A., Wang, L., Kreisberg, N. M., Jayne, J. T., & Liu, Y. (2022). Dynamics of di-2-ethylhexyl phthalate (DEHP) in the indoor air of an office. *Building and Environment*, *223*, 109446.
10. Kumawat, M., Sharma, P., Pal, N., James, M. M., Verma, V., Tiwari, R. R., Shubham, S., Sarma, D. K., & Kumar, M. (2022). Occurrence and seasonal disparity of emerging endocrine disrupting chemicals in a drinking water supply system and associated health risk. *Scientific Reports*, 12(1).
11. Gani, K. M., Rajpal, A., & Kazmi, A. A. (2016). Contamination level of four priority phthalates in North Indian wastewater treatment plants and their fate in sequencing batch reactor systems. *Environmental Science: Processes & Impacts*, *18*(3), 406–416.
12. Sarvaiya, V. N., Sadariya, K. A., Rana, M. P., & Thaker, A. M. (2014). Zebrafish as model organism for drug discovery and toxicity testing: a review. *Veterinary clinical science*, *2*(3), 31-38.
13. Yang, L., Ho, N., Alshut, R., Legradi, J., Weiss, C., Reischl, M., Mikut, R., Liebel, U., Müller, F., & Strähle, U. (2009). Zebrafish embryos as models for embryotoxic and teratological effects of chemicals. *Reproductive Toxicology*, *28*(2), 245–253.
14. Hill, A., Teraoka, H., Heideman, W., & Peterson, R. E. (2005). Zebrafish as a model vertebrate for investigating chemical toxicity. *Toxicological Sciences*, *86*(1), 6–19.
15. Test No. 236: Fish Embryo Acute Toxicity (FET) test. (2013). In *OECD guidelines for the testing of chemicals*.
16. Üstündağ, Ü. V., Ünal, İ., Ateş, P. S., Alturfan, A. A., Yiğitbaşı, T., & Emekli-Alturfan, E. (2017). Bisphenol A and di(2-ethylhexyl) phthalate exert divergent effects on apoptosis and the Wnt/β-catenin pathway in zebrafish embryos: A possible mechanism of endocrine disrupting chemical action. *Toxicology and Industrial Health*, *33*(12), 901–910.
17. Boran, H., & Terzi, S. (2019). Bis(2-ethylhexyl) phthalate induces DNA strand breaks and gene expression alterations in larval zebrafish *Danio rerio*. *Toxicology and industrial health*, *35*(8), 520–529.
18. Hamid, N., Junaid, M., Manzoor, R., Jia, P., & Pei, D. (2020). Prioritizing phthalate esters (PAEs) using experimental in vitro/vivo toxicity assays and computational in silico approaches. *Journal of Hazardous Materials*, *398*, 122851.

19. Muhammad, S., Zheng, Z., Pavase, T. R., & Guo, H. (2018). Long-term exposure of two plasticizers di (2-ethylhexyl) phthalate (DEHP) and acetyl tributyl citrate (ATBC): toxic effects on gonadal development and reproduction of zebrafish ("Danio rerio"). *Indian Journal of Geo-Marine Sciences*, *47*(4), 789–797.
20. Pu, S., Hamid, N., Ren, Y., & Pei, D. (2020). Effects of phthalate acid esters on zebrafish larvae: Development and skeletal morphogenesis. *Chemosphere*, *246*, 125808.
21. Lu, C., Luo, J., Liu, Y., & Yang, X. (2021). The oxidative stress responses caused by phthalate acid esters increases mRNA abundance of base excision repair (BER) genes in vivo and in vitro. *Ecotoxicology and Environmental Safety*, *208*, 111525.
22. Yumnamcha, T., Roy, D., Devi, M. D., & Nongthomba, U. (2015). Evaluation of developmental toxicity and apoptotic induction of the aqueous extract of Millettia pachycarpa using zebrafish as model organism. *Toxicological & Environmental Chemistry*, *97*(10), 1363–1381.
23. Dave, G., & Ruiqin, X. (1991). Toxicity of mercury, copper, nickel, lead, and cobalt to embryos and larvae of zebrafish,Brachydanio rerio. *Archives of Environmental Contamination and Toxicology*, *21*(1), 126–134.
24. Chikae, M., Hatano, Y., Ikeda, R., Morita, Y., Hasan, Q., & Tamiya, E. (2004). Effects of bis(2-ethylhexyl) phthalate and benzo[a]pyrene on the embryos of Japanese medaka (Oryzias latipes). *Environmental Toxicology and Pharmacology*, *16*(3), 141–145.
25. Zhang, Q., Ma, W., & Zhu, J. (2023). Combined toxicities of Di-Butyl phthalate and polyethylene terephthalate to zebrafish embryos. *Toxics*, *11*(5), 469.
26. Mu, X., Chen, X., Liu, J., Yuan, L., Wang, D., Qian, L., Qian, Y., Shen, G., Huang, Y., Li, X., Li, Y., & Lin, X. (2020). A multi-omics approach reveals molecular mechanisms by which phthalates induce cardiac defects in zebrafish (Danio rerio). *Environmental Pollution*, *265*, 113876.
27. Heideman, W., Antkiewicz, D. S., Carney, S. A., & Peterson, R. E. (2005). Zebrafish and cardiac toxicology. *Cardiovascular Toxicology*, *5*(2), 203–214.
28. Bakkers, J. (2011). Zebrafish as a model to study cardiac development and human cardiac disease. *Cardiovascular Research*, *91*(2), 279–288.
29. Wiegand, J. L., Avila-Barnard, S., Nemarugommula, C., Lyons, D., Zhang, S., Stapleton, H. M., & Volz, D. C. (2023). Triphenyl phosphate-induced pericardial edema in zebrafish embryos is dependent on the ionic strength of exposure media. *Environment International*, *172*, 107757.
30. Kuzmina, I. V. (2023). The yolk sac as the main organ in the early stages of animal embryonic development. *Frontiers in Physiology*, *14*.
31. Sant, K. E., & Timme-Laragy, A. R. (2018). Zebrafish as a model for toxicological perturbation of yolk and nutrition in the early embryo. *Current Environmental Health Reports*, *5*(1), 125–133.
32. Raldúa, D., André, M., & Babin, P. J. (2008). Clofibrate and gemfibrozil induce an embryonic malabsorption syndrome in zebrafish. *Toxicology and Applied Pharmacology*, *228*(3), 301–314.
33. Kodde, I. F., Van Der Stok, J., Smolenski, R. T., & De Jong, J. W. (2007). Metabolic and genetic regulation of cardiac energy substrate preference. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *146*(1), 26–39.
34. Li, Q., Wang, P., Chen, L., Gao, H., & Wu, L. (2016). Acute toxicity and histopathological effects of naproxen in zebrafish (Danio rerio) early life stages. *Environmental Science and Pollution Research*, *23*(18), 18832–18841.
35. Hill, A., Bello, S. M., Prasch, A. L., Peterson, R. E., & Heideman, W. (2004). Water permeability and TCDD-Induced edema in Zebrafish Early-Life Stages. *Toxicological Sciences*, *78*(1), 78–87.
36. Bagnat, M., & Gray, R. S. (2020). Development of a straight vertebrate body axis. *Development*, *147*(21).
37. Boswell, C. W., & Ciruna, B. (2017). Understanding Idiopathic scoliosis: a new zebrafish school of thought. *Trends in Genetics*, *33*(3), 183–196.
38. Bird, N. C., & Mabee, P. M. (2003). Developmental morphology of the axial skeleton of the zebrafish, Danio rerio (Ostariophysi: Cyprinidae). *Developmental Dynamics*, *228*(3), 337–357.
39. Dietrich, K., Fiedler, I. A., Kurzyukova, A., López-Delgado, A. C., McGowan, L. M., Geurtzen, K., Hammond, C. L., Busse, B., & Knopf, F. (2021). Skeletal biology and disease modeling in zebrafish. *Journal of Bone and Mineral Research*, *36*(3), 436–458.
40. Pamanji, R., Yashwanth, B., Bethu, M. S., Leelavathi, S., Ravinder, K., & Rao, J. V. (2015). Toxicity effects of profenofos on embryonic and larval development of Zebrafish (Danio rerio). *Environmental Toxicology and Pharmacology*, *39*(2), 887–897.

41. Çelik, E. S., Kaya, H., & Yılmaz, S. (2012). Effects of phosalone on mineral contents and spinal deformities in common carp (Cyprinus carpio, L. 1758). *Turkish Journal of Fisheries and Aquatic Sciences*, *12*(2).