ACUTE TOXICITY OF BISPHENOL A INDUCED PHENOTYPIC CHANGES ON ZEBRAFISH *(DANIO RERIO)* DURING EARLY DEVELOPMENT

Mai Ngo Thi[1, *], Ngoc Vo Thi Kim[2], Chi Do Hong Lan[3], Nga Le Phi[4]

[1]*Department of Occupational Hygiene and Health, Sub- Institute of Labour and Environment Protection in the South of Vietnam*

[2] *Ho Chi Minh University of Science, Vietnam National University, 227 Nguyen Van Cu, District 5, Ho Chi Minh City, Vietnam*

[3]*Institute of Environment and Resources, Vietnam National University, Ho Chi Minh City, Vietnam*

[4]*Ho Chi Minh University of Technology, Vietnam National University Ho Chi Minh City, 268 Ly Thuong Kiet street, District 10, Ho Chi Minh City, Vietnam*

[*]Email: *maingo25.7@gmail.com*

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ABSTRACT

Bisphenol A (BPA) has been considered as a weak environmental estrogen, as similar to estradiol that has a potential in stimulating some cellular responses and phenotype changes. In addition, the ecological impacts of BPA to aquatic organisms have been increasingly raised at environmental relevant concentrations that potentially may affect to human health at early–life. This study used 3-day old zebrafish larvae *(Danio rerio)* as a model for toxicological testing. The semistatic testing was conducted to investigate the effects of different BPA concentrations (5 mg/L, 6 mg/L, 7 mg/L, 8 mg/L and 9 mg/L) that induced morphorlogical and physiological changes during the early development. As the results, the $LC_{50\text{-}24hrs}$, $LC_{50\text{-}48hrs}$, $LC_{50\text{-}72hrs}$ and $LC_{50\text{-}96hrs}$ were determined as 9.503 mg/L, 8.688 mg/L, 7.328 mg/L and 6.669 mg/L, respectively. Phenotypic analysis revealed that toxicity caused cardiac edema. The result obtained from this research provided relevant information for environmental and human risk assessments.

Keywords: *Daniorerio*, zebrafish, bisphenol A, acute toxicity.

1. INTRODUCTION

Bisphenol A (BPA) is one of the highest produced chemicals worldwide. This compound is used in building block of polycarbonate plastics and component of epoxy resins that are used to line food and beverage containers [1]. There is an area of increasing interest that is the potential of BPA exposure which associates to obesity and metabolic complications [2].

Epidemiological studies have revealed that BPA may causes diseases and a number of cardiovascular risk factors such as hypertension, obesity, diabetes, metabolic syndrome and atherosclerosis [3 - 5]. BPA is also one of the most common endocrine disruptors in the environment. Although BPA was initially considered to be a weak environmental estrogen, more recent studies have demonstrated that this compound may act similar potency to estradiol in stimulating certain cellular responses. Moreover, emerging evidence suggests that BPA may influence multiple endocrine-related pathways.

Zebrafish *(Danio rerio)*, a model organism of vertebrate development and organogenesis, is receiving increasing attention as a model for human diseases, drug discovery and toxicology studies. Recently, zebrafish embryo and larvae are recommended to be the principal test organisms for endocrine disrupting compounds (ECDs) within the Organization for Economic Cooperation and Development (OECD) [6], thus zebrafish is a relevant model for investigating BPA toxicity. The availability of zebrafish in the large numbers, its small size and easy husbandry makes the zebrafish be a cost effective model than the other models for toxicological studies, for instance, rodent or rat. Owning to the conserved developmental program within vertebrates, fish and mammals share many similar developmental processes. Many of the genes or molecules with essential functions found in human such as those involved in developmental processes and toxicological responses are also found in zebrafish [7].

The larval stages of fish are sensitive to environmental stressors and the use of fish larva provides advantages for the testing and understanding of the toxic mechanism and environmental impacts of chemicals. Although quite large number of researches has been made on the BPA toxicity, less intention has been paid to the toxicity on the malformation. In this study, BPA was examined its toxicity on 3 day- post fertilization (dpf) (*Danio rerio)* larvae for molarity and morphology changes. The effect of BPA on early – life development has been little known so far. This larvae semistatic renew test, adopted from short term methods, is prior to be used for chronic toxicity in freshwater fish during development.

2. MATERIALS AND METHODS

2.1. Materials

Chemical tested: BPA (CAS no. 80-05-7) was purchased from Sigma Aldrich Co. (USA) and stock solution of 100mg/L was prepared by dissolving the chemical in 0.25% ethanol (Merk, Germany).

Zebrafish: wild- type adult zebrafish (*Danio rerio*) were initially purchased from a commercial source (Thu Duc District, Ho Chi Minh City, Vietnam). Female and male zebrafish were cultured in a glass aquarium aerated with a dissolved oxygen content approximate 90 % of saturation. The pH value was kept at 7.2 ± 0.2. The water temperature was 26.0 ± 0.2 ^{o}C with a light–dark period of 14 : 10 h. The fish were fed twice a day with dry flakes (TertraMin, > 48 % protein, UK). To ensure optimal water quality, any remaining food was removed daily. Embryos and larvae were obtained by natural mating with male: female ratio of 2:1 and raised in synthetic embryo water (Hank's Buffered Salt Solution (HBSS) [8]. Spawning and fertilization took place within 30 min when the light was switched on in the morning. A single mature female laid 100–300 eggs per day. The embryos were then collected from the aquarium and observed under a dissecting microscope for the experiments. After hatching, the 3 dpf larvae were exposed to different concentrations of BPA according to OECD guidance.

2.2. Determination of LC_{50} of bisphenol A for zebrafish larvae

Developing zebrafish were exposed to nominal concentration of 5 mg/L, 6 mg/L,7 mg/L,8 mg/L and 9 mg/L BPA with final concentration of 0,25 % (v/v) ethanol (solvent) in synthetic egg-water for 96 h (4 days) from 3 dpf (day post fertilization) onwards. Control fish were same condition, but without solvent. Fresh water with or without BPA was replaced a half volume of corresponding testing tank daily since half life time of this chemical in water was about 2.5 - 4.0 days [9].The larvae were in each of test tank and there were six tank replicates. No food was given during 4 day testing period, because the larvae itself utilized nutrients from the attached yolk sac. Endpoints used for assessing developmental toxicity were mortality and malformation (cadiac edema) after 24 h, 48 h, 72 h and 96 h exposure. The LC_{50} is the median lethal concentration at which 50 percent of a tested batch of fish were killed within a particular period of exposure [10] and it has been determined by EPA analysis program (Version 1.5) using Probit method. Cardiac edema (CE) is defined as a manifestation of congestive heart failure, due to increased venous and capillary pressures and often associated with renal sodium retention. Percentage of cardiac edema is the ratio of the number of cardiac edema zebrafish larvae to total of survived ones after exposure.

2.3. Observation of phenotype changes

Alive normal and abnormal zebrafish larvae exposed to BPA from 3 day post fertilization at 24 h, 48 h, 72 h and 96 h were observed under microscopic examinations (×100).

2.4. Statistical analyses

The difference among data of cardiac edema was analyzed using one way analysis of variance (ANOVA). The results are expressed as the mean ± standard error of the mean and values of $p < 0.05$ was considered to be statistically significant.

3. RESULTS AND DISCUSSION

3.1. The LC_{50} values

The LC_{50} values calculated with 95 % confidence limits are shown in Table 1. The 24 h, 48 h, 72 h and 96 h LC_{50} value was 9.503 mg/L (9.184 - 10.565), 8.688 mg/L (8.560 - 8.881), 7.681 mg/L (7.414 - 7.989) and 6.669 mg/L (5.654 - 7.607), respectively (Table 1). The mortality (%) was increased when BPA concentration increased. At the end of 96 h, all of the larvae exposed to 9 mg/L of BPA were dead (Fig. 1).

LC_{50} is the median lethal concentration at which 50 percent of a tested batch of fish were killed within a particular period of exposure [10]. The acute toxicity of BPA on aquatic organism has been studied using several species in the past decades. Previous acute studies on some other fish species such as Fathead minnow, *Oncorhynchusmykiss*, and *Cyprinodon variegates* showed 96 h LC_{50} values of 4.7, 3 - 4, and 7.5 mg/L, respectively [9]. The 96 h LC_{50} of 4.7 mg/L (4.0 - 5.5) obtained by using the static test method which was very similar of that given from semi static test method, 4.6 mg/L (3.6 - 5.4) as studied by Alexander et al. [11] on *Pimephalespromelas*. Yokota et al. [12] studied effects of BPA on the early life stages of Japanese Medaka (*Oryziaslatipes*) and obtained 96 h LC_{50} of 13.0 mg/L (11.6 - 14.7). ErtuğrulKankaya et al. [13] obseved the lower 96 h LC_{50} value for *Chalcalburnustarichi* larvae, 3.5 mg/L (2.8 -4.59).

The acute toxicity of BPA was conducted in variation of other testing models, for examples: cultured zebrafish liver cell line (ZFL) [14] and *Daphnia magna* [11]. *Danio rerio* embryo and larvae have been considered as suitable models for toxicity testing because of several advantages thus they were used for testing various chemicals, especially endocrine disrupting compounds (EDCs) [8]. There has been very few reports about BPA acute toxicity on *Danio rerio* embryo and larvae [6]. Chow et al. [15] reported a 96 h LC_{50} value of 8.041 mg/L (7.846 - 8.24) for BPA on 7 dpf *Danio rerio* larvae which is significantly higher than that obtained in our data but for 3 dpf *Danio rerio* larvae 6.669 mg/L (5.654 - 7.607), thus earlier stage of larvae is more sensitive to acute concentration of BPA. According to current EPA evaluation, BPA was slightly moderate toxicity to fish and invertebrates with LC_{50} or EC_{50} values of 1.1 - 10 mg/L [16].

Table 1. LC50 values and corresponding confidence intervals for zebrafish larvae exposed to BPA at 24, 48, 72 or 96 h

Time (hrs)	LC_{50} (mg/L)	95% CL (lower/upper)
24h	9.503	9.184 -10.565
48h	8.688	8.560 - 8.811
72h	7.681	7.414 -7 .989
96h	6.669	5.654 - 7.607

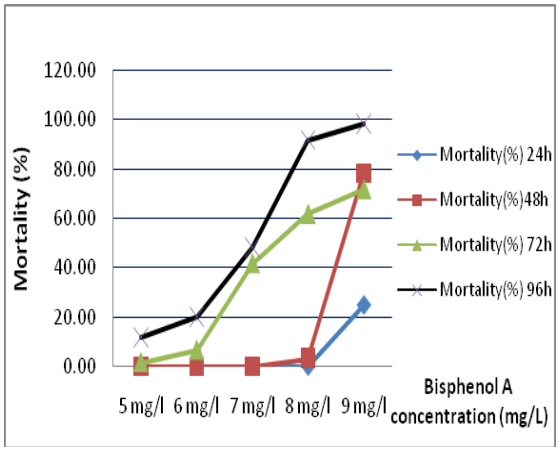

Figure 1. BPA for Molarity of zebrafish larvae exposed to 5 mg/L, 6mg/L, 7mg/L, 8 mg/Lor 9 mg/LBPA.

3.2. Phenotype analysis

There was little evidence of the abnormality of morphology in zebrafish exposed to BPA with high concentration in the short term of exposure. In this study, cardiac edema (CE) was observed at BPA concentration higher than 6 mg/L after 48 h. For 7mg/L, 8 mg/L and 9 mg/L, malformation was observed at 48 h as 3.33 %, 34.48 % and 69.23 %, respectively. For longer expose, at 72 h, CEwas observed at 6 mg/L BPA concentration (1.79 %), and that for 7 mg/L, 8 mg/L and 9 mg/L concentrations was 22.86 %, 56.52 % and 100 %, respectively ($p < 0.05$). Deformities were increased with BPA concentrations and exposure time increased. At 96 h, all of the surviving zebrafish larvae exposed to 8 mg/L and 9 mg/L was deformities as CE (Fig. 2, Table 2) ($p < 0.05$).

Ertuğrul et al. [13] observed cardiac edema in *Chalcalburnustarichi* larvae at 0.75, 1.5 and 3 mg/L BPA that included slowed heart rate and blood circulation, developmental arrest, yolk sac edema, spinal deformity, tail abnormalities, regression in the pigmentation, regressed swim bladder formation and delayed yolk sac withdrawal. Duan et al. [14] observed cardiac edema on zebrafish embryo after 72 h exposure to 20.87 mg/L BPA. Lam et al. [7] reported cardiac edema in developing zebrafish larvae treated with 1.5 mg/L and 4.5 mg/L BPA for 7 days from 3 hours post fertilization (hpf). In addition, fish with cardiac edema also appeared to have cranio-facial abnormality (broad–headed (branchycephalic) and lacks anterior lower jaw protrusion), problem with swim bladder development/inflation and apparent gastro – intestinal abnormalities and partial yolk sac re- absorption. New finding in our study is that BPA caused dose-dependent cardiac

edema (CE) was not observed at 5 mg/L BPA concentration during 4 days of exposure. BPA induce CE at concentration was slightly higher than that in Lam et al research [7] due to the difference of both the point beginning BPA exposure to zebrafish and used BPA concentration. That is likely early development of zebrafish should be considered for BPA toxicity testing.

Table 2. Cardiac edema (%) of zebrafish larvae at different concentrations of BPA after 24h, 48h, 72h or 96h exposure

	BPA concentration (mg/L)				
CE (%)	**5**	**6**	**7**	**8**	**9**
24h	0.00	0.00	0.00	0.00	0.00
48h	0.00	0.00	3.33	34.48	69.23
72h	0.00	1.79	22.86	56.52	100.00
96h	0.00	6.25	38.71	100.00	100.00

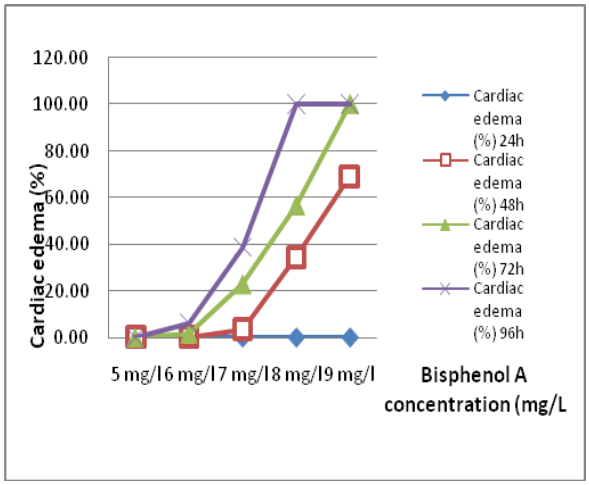

Figure 2. Cardiac edema (%) of zebrafish larvae at different concentrations of BPA after 24 h, 48 h, 72 h and 96 h exposure.

Figure 3. Normal and abnormal zebrafish larvae exposed to BPA. The arrow indicates cardiac edema.

4. CONCLUSION

Using zebrafish as a model for testing the toxicity of Endocrine Disrupting Compounds like bisphenol A in Vietnam has been at beginning stage. The results from our study indicate that early development stage of *Danio rerio* testing can provide more insights into BPA toxicity that is cardiac edema. The testing model here is suitable for not only acute but further chronic toxicity testing of BPA.

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REFERENCES

1. Yang J., Chan K. M. - Evaluation of the toxic effects of brominated compounds (BDE-47, 99, 209, TBBPA) and bisphenol A (BPA) using a zebrafish liver cell line, ZFL, Aquat. Toxicol**. 159** (2015) 138-147.
2. Newbold R. R. - Prenatal exposure to diethylstilbestrol (DES), Fertil.Steril. **89** (2008) e55-56.
3. Lang I. A., Galloway TS., Scarlett A., Henley W. E., Depledge M., Wallace RB., Melzer D. - Association of urinary bisphenol A concentration with medical disorders and laboratory abnormalities in adults, JAMA **300** (11) (2008) 1303-1310.
4. Lind PM., Lind L. - Circulating levels of bisphenol A and phthalates are related to carotid atherosclerosis in the elderly, Atherosclerosis **218** (1) (2011) 207-213.
5. Melzer D., Rice NE., Lewis C., Henley WE., Galloway TS. - Association of urinary bisphenol a concentration with heart disease: evidence from NHANES 2003/06, PLoS One **5** (1) (2010) e8673.
6. OECD - In No 47: Detailed review paper on fish screening assays for the detection of endocrine active substances, ENV/JM/MONO. **18** (2004).
7. Lam S. H., Hlaing M. M., Zhang X., Yan C., Duan Z., Zhu L. - Toxicogenomic and phenotypic analyses of bisphenol-A early-life exposure toxicity in zebrafish., PLoS One. **6** (12) (2011) e28273.
8. OECD – Test guide No. 234: Fish Sexual Development Test (2011).
9. Staples C. A., Dorn P. B., Kleck G. M., O'block S. T., Harris L. R. - A review of the environmental fate, effects, and exposures of bisphenol-A, Chemosphere. **36** (1998) 2149–2173.
10. US Environmental Protection Agency - Methods of measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms, 5th edition, WashingtonDC., USA, 2002, p.71.
11. Chen MY., Ike M., Fujita M. - Acute toxicity, mutagenicity, and estrogenicity of bisphenol-A and other bisphenols, Environ Toxicol. **17** (1) (2002) 80-86.

12. Yokota H., Tsuruda Y., Maeda M., Oshima Y., Tadokoro H., Nakazono A., Honjo T., Kobayashi K. - Effect of bisphenol A on the early life stage in Japanese medaka (*Oryziaslatipes*), Environmental Toxicology and Chemistry **19** (2000) 1925–1930.
13. Ertuğrul K., Burak K., AbdulahadD., İsmail Ç. - Toxicity of bisphenol a during the early life stages of *Chalcalburnustarichi* (Pallas, 1811), Fresenius Environmental Bulletin **24** (3A) (2015) 977-985.
14. Duan Z., Zhu L., Zhu L., Kun Y., Zhu X. - Individual and joint toxic effects of pentachlorophenol and bisphenol A on the development of zebrafish (*Danio rerio*) embryo, Ecotoxicol Environ Saf. **71** (3) (2008) 774-780.
15. Chow W. S., Chan W. K., Chan K. M. - Toxicity assessment and vitellogenin expression in zebrafish (*Danio rerio*) embryos and larvae acutely exposed to bisphenol A, endosulfan, heptachlor, methoxychlor and tetrabromobisphenol A, J. Appl. Toxicol. **33** (7) (2013) 670-678.
16. Alexander H. C., Dill D. C., Smith L. W., Guiney P. D., Dorn P. - Bisphenol a: Acute aquatic toxicity, Environmental Toxicology and Chemistry **7** (1988) 19–26.