TOXICITY OF BISPHENOL A ON THE GROWTH OF ZEBRAFISH EMBRYOS

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Abstract: In order to evaluate the toxicity of the bisphenol A (BPA) on the growth of zebrafish embryos, fertilized eggs were exposed to the concentration of 2.00, 4.00, 6.00, 8.00, 10.00, 15.00, 18.00, 22.00 and 25.00mg/L BPA for 72 h at 26±1℃. The results revealed that the sublethal toxicological endpoints induced by BPA were: delayed hatch> blood balk> cyst> altered axial curvature and tail malformation. The median embryo lethal concentration (LC50) after 24 h was 16.36 mg/L. We concluded that the BPA toxicity on zebrafish embryos were caused before 8h exposure and it was not the result of long-term accumulation. Therefore, BPA maybe cause altered gene expression at the early stage of zebrafish embryos. In the further studies, we will use the technology of genetic chips to look for the toxic mechanism of BPA.

Key words: Bisphenol A; Zebrafish embryos; Toxicity

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Bisphenol A (BPA) is a chemical estrogen-like substance with properties that are of environmental concern. It is widely used in the chemical industry to manufacture epoxy- and polyester-styrene resins. It has been reported that BPA ranges between 0 and 33μg in each plastic cup[1]. After a two-week exposure to 0.5% bisphenol A it has been reported that disattachments between sertoli cells and spermatogonia were observed while spermatogonia were arranged in disorder and displacement of spermatogonia away from the basement membrance of seminferous tubules as well as flocculated chromatins of nuclei in sertoli cells and spermatogonia occurred[2]. At high exposure level (60.00mg/L), BPA could retard the growth and cardiac tube differentiation of visceral yolk sac (VYS), and induce various embryonic defects, including an abnormal neural system, arch, optic, flexion and small limb bud, etc.[3].

In professional production and in daily life, absorption of large amounts of bisphenol A through the skin resulted in extensive damages to kidney, liver, spleen, pancrea and lung[4]. Because of its wide usage in plastics, BPA has caused great environmental pollution. Studies have testified that BPA is potentially toxic to embryos and causes genetic defects. An exposure to human estrogen-sensitive MCF-7 breast cancer cells produces an increase in cell proliferation and changes in the lever of progester-one receptors[5]. BPA can also be converted to DNA binding metabolites in vitro[6].

Zebrafish have merits as test species because of simple breeding technology, short growth period, high fecundity and transparent eggs. Therefore, zebrafish is a perfect model to study the developmental growth of vertebrates[7,8]. Zebrafish embryos have become an effective test material to substitute adult fish in bioassay testing.

1 Materials and methods

1.1 Fish Zebrafish were purchased from a local ornamental fish supplier (Tianjin, China). All adult fish (female: male ratio = 1∶2) were maintained indoors at an ambient temperature of about 26℃ with a natural dark/light cycle of 10/14 hours in the zebrafish aquarium facility ($80cm^3 \times 40cm^3 \times 50cm^3$), and they were fed with frozen

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chironomid larvae from an uncontaminated source, twice daily. Charcoal filtered tap water was used for breeding the fish. The volume of the water in the tank was about 100L, and the number of the fish in the tank was about 200. The oxygen saturation was maintained above 80%. The temperature of the water was controlled by an automatic heat stick and measured by a thermometer. The fish were acclimated for about one month before being used.

1.2 Chemicals Bisphenol A (2, 2-di (4-hydroxyphenyl) propane) was purchased from Fuchen Chemical Company (*Tianjin, China*; purity > 98.0 %). All other chemicals including ethanol were reagent grade and purchased from a commercial source.

The water used in this experiment was MiliQ water (Miliport Corp.) or synthetic water. The chemical composition of synthetic water included 12.95mg/L $NaHCO_3$, 1.15mg/L KCl, 24.65mg/L $MgSO \cdot 7H_2O$, and 58.80 mg/L $CaCl_2 \cdot 2H_2O$.

Bisphenol A was dissolved in the synthetic water together with a little ethanol as cosolvent. The concentration of ethanol in the actual exposure solution should be no more than 0.01%. The test concentrations of BPA were 0.00, 2.00, 4.00, 6.00, 8.00, 10.00, 5.00, 18.00, 22.00 and 25.00mg/L.

1.3 Equipment The equipment needed for the study included: (a) inverted microscope, (b) NUNC 24-well multiplates, (c) air and light conditioned room, (d) temperature and light controlled aquarium, (e) pH-meter, (f) oxygen meter, (g) automatic heat stick, (h) thermometer, (i) self-adhesive foil to cover multiplates, (j) glass spawn trap (L×W×H = 30cm×18cm×6cm), and (k) glass vessels.

1.4 Exposure The fertilized eggs were separated from the non-fertilized eggs under a stereo microscope and placed in wells of multiplates. There were totally 24 eggs in one 24-well plate for one test concentration. For 20 wells, each well has one fertilized egg placed in respectively with 2mL test solution. The remaining 4 wells of each plate were filled with 2mL dilution water and one egg per well as internal contrast. Then all the multiplates with eggs were sent to the air conditioned room. Water temperature was maintained at 26±1 ℃ in test chambers. Toxicity tests were designed to determine which concentration, developmental stage, and exposure duration induced specific teratogenic defects. Therefore, exposure was designed with three ways: firstly, early cleavage (immediately after fertilization, 0hpf) exposure; secondly, gastrula (8h post-fertilization, 8hpf) and segmentation (24hpf) exposure; finally, exposure started at 0hpf for 8 hours and then the embryos were picked out and maintained them in the dilution water (8h paused exposure).

1.5 Statistical analysis Data from microscopic examinations of the embryos were categorized according to types and severities of effects, and then the median embryo lethal concentration and the median effect concentration were calculated from the statistics. All experiments were repeated three times respectively. Student's t-test was used for mean separation analysis, and data were illustrated by means of standard deviation (S.D.). To evaluate dose-response relationships, post hoc tests for linear trend used the Origin software. Comparisons tests between groups were carried out by one-way ANOVA. A p value of < 0.05 was considered statistically significant different.

2 Results

Firstly, at the 0hpf exposure, blisters were brought after 4h exposure, and the median effect concentration (EC_{50}) of it was 13.00±0.60mg/L (Tab. 1), and the lethal concentration (LC_{50}) after 24h was 16.36±0.40mg/L whereas all the embryos were dead when exposed at 25.00 mg/L. Exposure of zebrafish embryos to sublethal concentrations of BPA consistently caused several types of morphological defects. The duration of the exposure, developmental stage, and concentrations, all affected the frequency and severity of teratogenic defects. The primary effects were blood balk in the circulatory system, cyst (Fig. 1 (C, D)) and delayed hatch. The median effect concentrations (EC_{50}) for adverse effects (mortality and developmental defects) were observed (Tab. 1). Meanwhile, high concentrations of BPA treated embryos showed altered axial curvature and tail malformations (Fig. 1 (E, F)). These results revealed that the sublethal toxicological endpoints induced by BPA were: delayed hatch > blood balk > cyst > altered axial curvature and tail malformation.

Tab. 1 The observed median effect concentrations (EC_{50}) for adverse effects (mortality and developmental defects) of 0hpf exposure

Toxicological endpoints	4h-blisters brought	32h-blood block	72-delayed hatch	72h-cyst
EC_{50}	13.00±0.60mg/L	15.00±0.80mg/L	13.00±0.50mg/L	NA[a]

NA[a]-None applied data. While the concentration of BPA was 15.00mg L^{-1}, the percent of cyst exhibited by zenrafish embryos of 0hpf exposure was 17.64%.
[b]None abnormal effect was shown in blank control groups

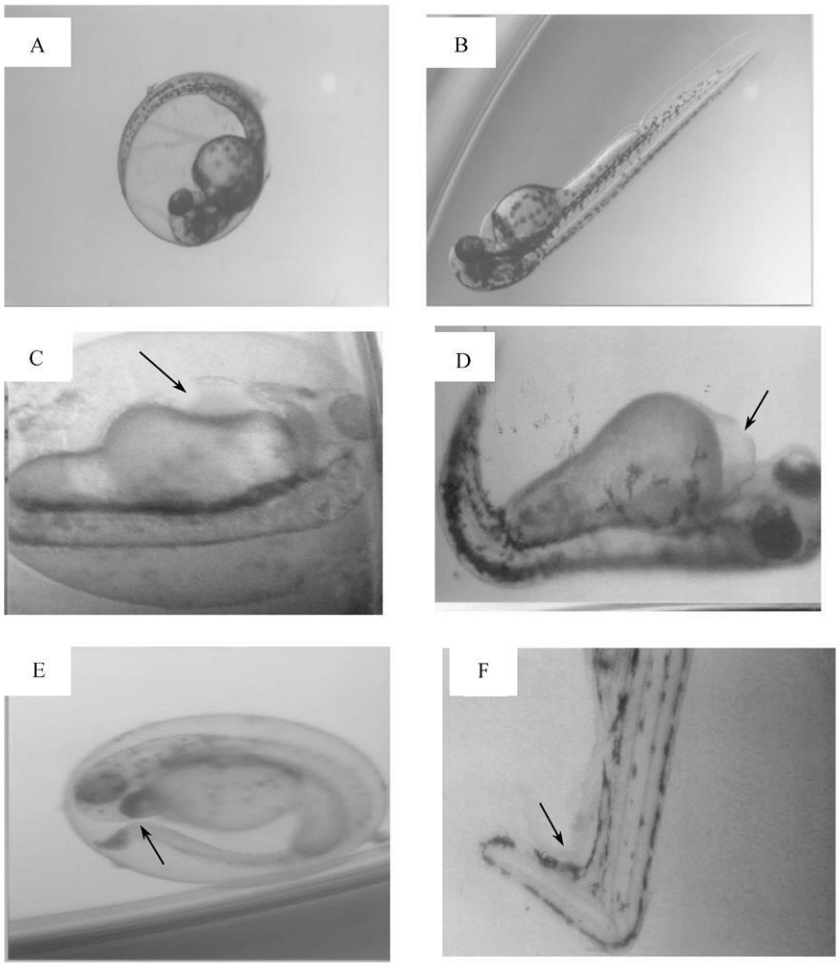

Fig. 1 Observed normal and abnormal embryo and fish. (A) 48h normal embryo, in contrast solution; (B) 72h normal fish after hatched out, in contrast solution; (C) 48h cyst in the embryo, 8hpf exposure, 15.00mg/L BPA; (D) 72h cyst after hatched out, 8hpf exposure, 15.00mg/L BPA; (E) 72h tail malformationsin the embryo, 8hpf exposure, 20.00mg/LBPA; (F) 72h tail malformations after hatched out, 8hpf exposure, 20.00mg/LBPA

Secondly, zebrafish embryos were incubated in the above-mentioned concentrations of BPA from 8hpf (8 h post fertilization) and 24hpf contrast to 0hpf. Embryos exposed after an age of 32 hours showed that while at 0hpf various toxicity effects occurred, none was noted at later starting points of exposure. At 0hpf and 8hpf exposure, the effects of delayed hatch and cyst at 48h and 72h were both much more pronounced than those starting at 24hpf exposure. There was no difference between 0hpf and 8hpf at lethal and sublethal toxicity, while 24hpf had much lower effects (Tab. 2).

Tab. 2 Effects of BPA on embryonic development and subsequent malformations after various postfertilization starting times to test concentrations: 0hpf = immediately after fertilization, 8hpf = 8 hours post-fertilization and 24hpf = 24 hours post fertilization

Toxicity endpoints		0hpf exposure	8hpf exposure	24hpf exposure
32h		While at 0hpf various toxicity effects occured, none was noted at later starting points to exposure		
48h	Hatch rate	None Observed	None Observed	EC_{50}= 18.00±2.00mg/L[b]
	Cyst	Observed	None Observed	None Observed
72h	Delayed hatch rate	13.00±0.50mg/L	Similar to the 0hpf exposure[a]	EC_{50}= 25.00±1.50mg/L[b]
	Cyst	While at 15.00mg/L, 17.64% observed	While at 15.00mg/L, 5.26% observed	Observed only after 25.00mg/L

[a]While BPA concentration was 10.00mg/L, hatch rate was 22.00%;

[b]Strictly, it should not be called EC_{50} here. We just mean the BPA concentration while the rate was 50.00% for conversion

Finally, zebrafish embryos were exposed at 0hpf but paused after 8h contrast to the 72h persistent incubation. The differences between the above two treatments were not obvious after 32h development stage. The EC50 of the two sublethal effects (blood balk, and delayed hatch) at 8h paused exposure were 19.00±0.50 and 19.00 ±1.60mg/L BPA (Fig. 2). The difference between these treatments above was analyzed by one-way ANOVA. The p value of 32h blood balk was 0.025 while that of 72h delayed hatch rate, was 0.016. Therefore, it was found that the effects of 8h paused exposure was significantly different with 72h sustaining exposure, which meant the toxicity of BPA was greatly decreased at 8h paused exposure.

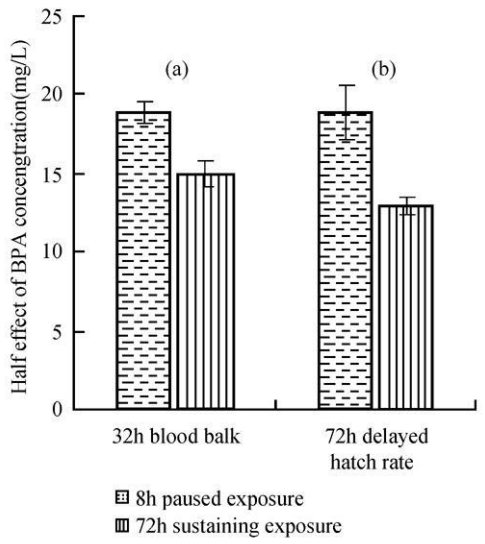

Fig. 2 Effects of BPA in 8h paused exposure and 24h sustaining exposure. (a) 32h blood balk, $p = 0.025 < 0.05$; (b) 72h delayed hatch rate, $p = 0.016 < 0.05$. According to the p values, it was found that the effects of 8h paused exposure were significantly different with 72h sustaining exposure, which meant the toxicity of BPA was greatly decreased at 8h paused exposure

3 Discussion

Zebrafish embryos proved to be sensitive bioassay organisms for assessing the toxicity of environmental pollutants. The embryonic development of zebrafish has been divided into six major stages (early cleavages, blastula, gastrula, segmentation, pharyngula, and hatching) and each is defined by a variety of developmental events. The test exposures, beginning at different developmental stages and continuing for various periods of time, identified which stages were most vulnerable to chemical induced abnormalities. In this study, we found bisphenol A has profound effects on the developmental stage of zebrafish embryos. The experiment of 8-hour paused exposure revealed that it had nearly no difference with the 72-hour continued exposure at lethal and sublethal toxicity level. While the embryos were exposed after 8 hours, the toxicity of BPA was nearly the same with 0hpf exposure, but it would decrease greatly at 24hpf exposure. Therefore, we can conclude that BPA may cause altered gene expression at the early stage of zebrafish embryos, but it is not the result of accumulation.

However, there are two shortcomings in this study. One is that BPA is a kind of surfactant. Because its surface tension is much higher than that of the water, when it is dissolved in the water, it will mainly act at the water-substance inter-surface. That means the actual concentration of BPA at the water-surface interface is higher than in the bulk of the test water. However, the zebrafish embryos are always at the bottom of the multiplate wells. Therefore, the concentrations of BPA could not reach the

entire surface area of the eggs. Another aspect is that BPA is also a kind of organic substance; it is easily adsorbed by the plate walls and bottoms as well as at the egg surface. This rapid absorption will reduce the real concentration of BPA in the water, but absorption on the egg surface will also prevent some of the test substance to directly reach the embryo inside the egg. One way to study to true concentration acting at the test organism could be to use the GC-MS technology to calibrate the concentration of BPA in the water and embryos at each developmental stage to revise our results.

In summary, our findings are significant because we demonstrate that the environmental estrogen-like chemical, bisphenol A, has effect on the zebrafish embryos at the early growth stage, but it is not the result of accumulation (The parent generation has not been exposed). Although it is unsure that whether bisphenol A alters the gene expression, our results may also provide markers for the further study on the development of the animals and humans in response of exposure to environmental estrogen-like chemicals. In the further studies, we look forward to the technology of genetic chips to look for the toxic mechanism of BPA.

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