Chemosphere 221 (2019) 727-734



Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Determination and reduced life expectancy model and molecular docking analyses of estrogenic potentials of 17β -estradiol, bisphenol A and nonylphenol on expression of vitellogenin gene (*vtg1*) in zebrafish



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Chemosphere

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HIGHLIGHTS

- Binary mixtures of E₂, BPA and NP have weak synergistic effects on hepatic *vtg1* gene of zebrafish.
- The RLE model for lethal toxicity was extended to study the effect of exposure time on EC₅₀ of *vtg1* levels.
- A new interpretation for interaction potential of binary mixtures of E₂, BPA and NP was given through molecular docking.

ARTICLE INFO

Article history: Received 28 October 2018 Received in revised form 13 January 2019 Accepted 15 January 2019 Available online 16 January 2019

Handling Editor: David Volz

Keywords: Reduced life expectancy model Molecular docking analysis Estrogenic potential Bisphenol A Nonylphenol Zebrafish

G R A P H I C A L A B S T R A C T



ABSTRACT:

This study determined and evaluated the estrogenic potentials on hepatic vitellogenin gene (vtg1) of adult male zebrafish which were exposed to low level concentrations (ng/L-µg/L levels) of individual and binary mixtures of 17β-estradiol (E₂), bisphenol A (BPA) and nonylphenol (NP) through the use of reduced life expectancy (RLE) model and molecular docking analysis. The order of in vivo estrogenic potentials of individual and binary exposure of E₂, BPA and NP was as follows: $E_2+BPA > E_2 > E_2+NP > BPA > BPA + NP >>>NP. Binary mixtures of E₂, BPA and NP had weak synergistic effects under the exposure concentration ranges. With the expression of hepatic vtg1 gene, the hepatic toxicity was analyzed by using the RLE model. All plots of the linear RLE model had negative slopes indicating that <math>EC_{50}$ was negatively correlated with the natural logarithm of exposure time ($InET_{50}$). The RLE model analyses can be useful to evaluate the exposure time effects of zebrafish by using EC_{50} as toxicity endpoint. Molecular docking analysis revealed that the interaction potential of E_2 (Binding energy: -10.1 kcal/mol) for the zebrafish ER α -LBD was the most potent (stable), followed by BPA (-8.0 kcal/mol) and NP (-6.8 kcal/mol). Molecular docking analysis can be useful to understand interactive effects of E_2 , BPA and NP.

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https://doi.org/10.1016/j.chemosphere.2019.01.093 0045-6535/© 2019 Elsevier Ltd. All rights reserved.

1. Introduction

Estrogenic effects of environmental chemicals on organisms and

humans have become a serious health risk concern around the world (Yamamoto et al., 2001; Renou et al., 2008; Clarke et al., 2015). It has been reported that xenoestrogens, such as bisphenol A (BPA) and nonylphenol (NP) are widely found in water, soil, municipal sewage and landfill leachate (Soares et al., 2008; Villeneuve et al., 2012). Among them, landfill leachate can infiltrate the groundwater and therefore cause environmental pollution (Liu et al., 2015). Studies have indicated that both BPA and NP were detected at the same time in groundwater contaminated by landfill leachate (Buszka et al., 2009). Due to their low water solubilities and high octanol-water partition coefficients, xenoestrogens tend to be relative stable in the environment (Meylan et al., 1999; Ying et al., 2002). Even at the low concentrations (ng/L-µg/L levels), they can have serious consequences for human and wildlife endocrine systems (Hernando et al., 2006). Therefore, it is important to study the estrogen effects of low concentrations of BPA and NP on organisms and to evaluate the potential risks of landfill leachate to the environment.

The vtg genes (mainly vtg1) produce vitellogenin (VTG) in zebrafish hepatocytes by transcriptional expression, which can be used as a biomarker to appraise the effect of endocrine disrupters (Teta and Naik, 2017; Chen et al., 2018). Research indicates that individual exposures of chemical induce the expression of vtg1 gene and the plasma VTG in male zebrafish, and, the vtg1 gene can response quickly to exposure (Van den Belt et al., 2003; Jin et al., 2009; Yamaguchi et al., 2015). Studies have shown that the mixture of xenoestrogens has combined effects on the zebrafish vtg1 gene (Urbatzka et al., 2012), but the nature of the combined effects of the mixtures based on the low concentrations (ng/L-µg/L levels) and the effect of the length of the exposure periods, as well as the contributions of the molecular structure of each compound in the mixture to the combined effect have rarely been reported.

BPA and NP often produce estrogen effects on organisms in low concentrations and long periods, so it is necessary to evaluate the impact of this situation over the life expectancy of organisms. This exposure time effect is of significance in environmental risk assessment. Verma et al. (2013) based on the influence of exposure time, established a reduced life expectancy (RLE) model to study the time response relationship in lethal toxicity. The RLE model established a relationship between the internal lethal concentration (ILC₅₀) and the natural logarithm of exposure time $(lnLT_{50})$ with the normal life expectancy of the organism as a limiting point (Connell et al., 2016). The LC_{50} (lethal concentration) and EC_{50} (effect concentration) are often used in toxicity endpoints in various tissues, both of which are related to nominal concentrations and logKow (octanol partition coefficient) (Verma et al., 2014). By analogy, the RLE model to analyze the relationship between LC_{50} and exposure time can be extended to describe the relationship between EC₅₀ and exposure time.

Molecular docking analysis is based on a new heuristic search algorithm, which can mimic the three-dimensional structure of the binding of the endocrine disruptor ligand to the ER α -ligand binding domain (ER α -LBD) (Thomsen and Christensen, 2006). Although various studies have compared the affinity difference between BPA and NP with natural estrogen E₂, they are generally based on data from in vitro experiments with recombinant yeast methods (Routledge and Sumpter, 1996). They are unable to obtain information such as the difference between the type of intermolecular interactions and the key amino acid residues used to stabilize the conformation obtained by molecular docking. On the other hand, the structure-response of BPA and NP binding with ER α should give a reasonable explanation for the concentration-response and time-response of the xenoestrogens.

In the present study, adult male zebrafish were exposed to low concentrations $(ng/L-\mu g/L \text{ levels})$ of BPA, NP together with the

natural estrogen E_2 and their binary mixtures. The hepatic vtg1 gene was used as a biomarker for exploring the concentrationtime-structure response relationship. The effect of exposure periods on extrogenic effects were evaluated by using the RLE model, and molecular docking was used to evaluate the contribution of the molecular structure of each compound in the mixture. The research results are of significance for the risk assessment of endocrine disruptors in the environment.

2. Materials and methods

2.1. Experimental animals and reagents

Adult male zebrafish (approximately four months old) were exposed in semi-static system (20 L glass aquaria) using active carbon treated tap water. Renewals were conducted for each 3 days, with 100% of the water refreshed. Throughout the experiment, the water temperature was maintained at 28.5 ± 0.2 °C, the dissolved oxygen remained at 6.5 ± 0.3 mg/L, the pH was 7.6 ± 0.5 , and the photoperiod was 14 h light and 10 h dark. The average weight and length of zebrafish were 0.190 ± 0.003 g and 2.220 ± 0.005 cm. The zebrafish were fed twice daily with estrogen-free frozen brine shrimp at 4% (w/w) body weight. The zebrafish were housed in the above-mentioned condition for at least 15-day during the pre-exposure period. E₂ (98% purity), BPA (98% purity) and NP (98% purity) were purchased from Sigma–Aldrich.

2.2. Single compound exposure experiments

The zebrafish were exposed to each compound individually at the following conditions, E_2 (2.5, 5, 10, 25, 50 ng/L), BPA (25, 50, 100, 250, 500 µg/L), or NP (0.25, 0.5, 1, 2.5, 5 µg/L) via waterborne, semistatic renewal exposure for five exposure periods (3, 6, 9, 12 and 15 days). The exposure concentrations were set with reference to the detection levels of BPA and NP in landfill leachates. The concentrations of BPA in landfill leachates of Japan ranged from 1.3 to 17,200 µg/L (Yamamoto et al., 2001); The concentrations of NP in landfill leachates collected in Sweden ranged from 0.1 to 7.3 µg/L (Kalmykova et al., 2013). E₂ has a short half-life and it is not the main pollutant in the landfill leachate. Its exposure concentrations were set with reference to the detection levels of E₂ in sewage treatment plants. The concentrations of E₂ in influents of Japanese STPs ranged from 3.2 to 55 ng/L (Nasu et al., 2001).

2.3. Binary exposure experiments

Concentrations of binary mixtures of E_2 , BPA and NP were added at proportions of the average of each respective EC_{50} of individual exposure at the five exposure periods (3, 6, 9, 12 and 15 days). In order to unify the TU ratio of individual compound in combined exposure solutions by the Toxic Unit (TU) method (Son et al., 2016), we used the EC_{50} of NP of 5 μ g/L, and the nominal concentration of NP in combined exposure solutions was obtained accordingly. Five different concentrations for binary mixtures of E_2 , BPA and NP were prepared at concentrations of $\sum 0.75$ TU, $\sum 1.0$ TU, $\sum 1.25$ TU, $\sum 1.5$ TU, and $\sum 1.75$ TU (their corresponding sums of toxic unit, Σ TU) by applying the Toxic unit (TU) approach. The data in columns 2, 3, 4, 5, 6 and 7 of "Table 2 in the results section" correspond to the nominal concentration of single compound in combined exposure solutions. The data in columns 8 of "Table 2" correspond to the nominal concentration of the binary mixture.

2.4. Experimental conditions

One tank was used for each concentration. Control tank

Table 1

Logistic probit analysis of EC₅₀ values of *vtg1* mRNA expressions and nominal concentrations (or sums of toxic units obtained from binary mixture) of E₂, BPA, E₂+BPA, E₂+NP and BPA + NP for 3, 6, 9, 12 and 15 days.

Endocrine disrupters	Exposure time (day)	EC ₅₀	95% lower confidence limit	95% upper confidence limit
E_2 (ng/L)	3	24.01	21.35	27.10
	6	26.54	17.64	41.42
	9	22.72	5.34	50.50
	12	19.07	10.74	31.67
	15	18.49	9.69	31.49
BPA (µg/L)	3	244.99	118.89	465.61
	6	248.11	69.33	466.79
	9	193.88	126.77	278.73
	12	183.26	113.34	290.99
	15	166.29	105.21	264.01
E ₂ +BPA (TU)	3	1.169	0.440	1.572
	6	1.253	0.695	1.436
	9	1.210	0.626	1.537
	12	1.128	0.681	1.503
	15	1.115	0.843	1.352
$E_2 + NP (TU)$	3	1.122	0.076	1.468
	6	1.188	0.680	1.726
	9	1.169	0.655	1.676
	12	1.115	0.594	1.530
	15	1.077	0.704	1.362
BPA + NP (TU)	3	1.039	0.293	1.448
	6	1.151	0.193	2.048
	9	1.093	0.652	1.432
	12	1.070	0.631	1.385
	15	1.054	0.533	1.390

Table 2

Equivalent toxicity of concentrations of binary mixtures of E₂, BPA and NP (μ g/L) and their corresponding sums of toxic units (\sum TU) obtained from individual exposure toxicity studies.

Toxic unit	Mixture combination						
TU (x,y)	E2+BPA		E2+NP		BPA + NP		∑TU ^a
0.375 0.5 0.625 0.75 0.875	0.008 0.011 0.014 0.017 0.019	77.7 103.7 129.6 155.5 181.4	0.008 0.011 0.014 0.017 0.019	1.9 2.5 3.1 3.8 4.4	77.7 103.7 129.6 155.5 181.4	1.9 2.5 3.1 3.8 4.4	0.75 1 1.25 1.5 1.75

containing ethanol at 0.01% were set up concurrently with test tanks. The nominal concentration of E_2 , BPA, NP and their binary mixtures were re-added into the test tanks after the water refreshed for each 3 days, to maintain the concentration to be the same as those at the beginning of the experiments. At the end of each exposure period (3, 6, 9, 12 and 15 days), eight fishes were randomly selected from the aquarium; six fishes were divided into three parallel controls and two fish for backup; fish were anesthetized by immersing in tricaine methane sulfonate (MS222) and then dissected, and livers were removed. The livers were frozen immediately in liquid nitrogen and dry ice until the RNA was isolated.

2.5. Zebrafish hepatic vtg1 mRNA analysis

Total RNA was extracted from the livers of zebrafish from each of the exposed groups and the control group, according to the manufacturer's protocol. The total RNA of zebrafish liver tissue extracted by TRIzol method was measured by UV spectrophotometer. The A260/A280 ratio was 1.91 ± 0.07 , between 1.8 and 2.0, without protein and DNA contamination. Syntheses of cDNA and gene expression were conducted according to the methods described previously (Woods and Kumar, 2011). The reverse transcribed cDNA template was amplified to detect the template quality of the reverse transcription. Amplification reactions were carried out in duplicates with qPCR using SYBR Green detection according to protocols established by the manufacturer. The PCR primers were designed with Primer Premier 5.0: vtg1 (GenBank Number of AF406784.1) forward primer 5'-AACGAACAGCGAGAAAGAGATTG-3'; reverse primer 5'-GATGGGAACAGCGACAGGA-3'; β-actin (Gen-Bank Number of AF057040.1) forward primer 5'-GTGCTGTTTTCCCCTCCATTGTTG-3'; β-actin reverse primer 5'-GTGTCATCTTCTCTCTGTTGGCTTT-3'. The present study analyzed the melting curves of β -actin PCR product to verify that the designed primer worked well. The quantification of target gene expression was based on the comparative cycle threshold (Ct) method. The β -actin expression was used as internal control for all expression data normalization.

2.6. Reduction life expectancy (RLE) model

The linear RLE model (Verma et al., 2015) was developed with the use of the concept of reduction in life expectancy and the model equation is given below:

$$LC_{50} = \left[(\ln NLT_{50}) - \ln LT_{50} \right] / d \tag{1}$$

or

$$LC_{50} = -\ln LT_{50} + b$$
 (2)

where LC_{50} is the lethal concentration, LT_{50} is the exposure time, NLT_{50} is the normal life expectancy of the organism, d is a constant, a is 1/d and b is $lnNLT_{50}/d$.

The nonlinear RLE model (Verma et al., 2014) was developed based on the concept that the curvature could be described by taking the LC_{50} to an exponent value v. The nonlinear RLE model can be expressed as:

$$\ln(LT_{50}) = -a(LC_{50})^{\nu} + b \tag{3}$$

where a is a slope coefficient, b the intercept related to NLT and v the exponent for LC_{50} . The value of v is nonlinearity constant.

2.7. Molecular docking simulations

The homology model for the zebrafish ERα-LBD and the analysis of the interaction potential between E2, BPA and NP and the zebrafish ERa-LBD were described previously (Webb and Sali, 2014). The crystal structures of the human ER_α-LBD (Estrogen receptor alpha ligand binding domain) were obtained from the Protein Data Bank (PDB). The human $ER\alpha$ -LBD chain B (PDB ID: 3DT3) as template were constructed a model of homologous ERa-LBD, and modeling structure was optimized by the molecular mechanics method. The assessment results of the zebrafish ER_α-LBD modeling showed that no amino acids fell into outlier regions, indicating that the conformation of all amino acids was reasonable. The protein of PDB code 1JJ4 (E₂) and 3UU7 (BPA) were generated by AutoDock Tools (Huey and Morris, 2003), while the small molecule NP structure were drawn by chemoffice and optimized using the MM2 force field. Docking produces a small molecular conformation file and analyzes it with PyMol (DeLano, 2002). The lowest affinity conformation was selected as the final solution.

Automated docking calculations were carried out by AutoDock Vina (Trott and Olson, 2010) to explore the binding modes between ER α and compounds. The crystal structure of zebrafish ER α -LBD (GenBank ID: AAK16740.1) was available in National Center for Biotechnology Information (NCBI). The amino acid sequences of zebrafish ER α -LBD were aligned with those of compounds to yield a readily superimposable three-dimensional model. It was calculated that the predicted binding affinities (kcal/mol) on the scoring function of AutoDock Tools (Huey and Morris, 2003). For each compound, eight independent docking runs were performed and the one with the lowest binding affinities was chosen for analysis. By the program PyMol (DeLano, 2002), the intermolecular interactions between ER α -LBD and compounds were visualized.

2.8. Statistical analysis

Prism software (GraphPad-Prism Software Inc., San Diego, CA) was used to analyze the statistical significance between the hepatic *vtg1* mRNA expression and the exposure concentration and time induced by individual and combined exposure. Two-way ANOVA was performed to detect statistically significant differences between any groups. Values were considered significant when P < 0.05. The EC₅₀ values of hepatic *vtg1* mRNA levels at the 3, 6, 9, 12 and 15 days were analyzed using SPSS software (Inc, 1994). Since the exposure time was only 15 days, the data sets available for individual and combined exposure tests were used to evaluate the linear relationship between EC₅₀ and lnET₅₀ with the linear RLE model as expressed in Equation (4). The EC₅₀ at the 3, 6, 9, 12 and 15 days was plotted against lnET₅₀ and linear regression analysis were used to obtain the regression equation and correlation coefficient using Origin software (Microcal Software Inc., Northampton, Massachusetts, USA). Results are shown as mean ± standard error of the mean (S.E.M), unless otherwise indicated.

3. Results

3.1. Relative mRNA levels of zebrafish hepatic vtg1

To determine the response of the *vtg1* gene and EC₅₀ values to individual and binary exposure of E₂, BPA and NP at the zebrafish levels, the relative mRNA levels of *vtg1* by q-PCR analysis were measured. Fig. 1 shows a dose-dependent increase of *vtg1* mRNA expression occurred in zebrafish following exposure to E₂, BPA, NP and their binary mixtures for 3, 6, 9, 12 and 15 days. The relative mRNA levels of *vtg1* in the E₂+BPA (\sum 1.75TU) (Fig. 1D) treated groups for 15 days were about 780 folds higher than that in the



Fig. 1. Dose-dependent expression of hepatic *vtg1* in adult male zebrafish exposed to various concentrations of E₂, BPA and NP, and their binary mixtures for 3, 6, 9, 12 and 15 days. Data normalized to housekeeping gene of β -actin. Fold change of dates are presented as mean \pm SEM (n = 3) relative to controls. The baseline (y = 1) indicates the level of control (0.01% ethanol). Statistically significant differences are shown by an asterisk, * for p < 0.05, ** for p < 0.01 and *** for p < 0.001. Relative expression levels of *vtg1* are plotted on a log scale (except for NP in Fig. 1C).

control group (P < 0.001). Compared with the control group, the vtg1 mRNA levels increased significantly in the 5 µg/L of NP (Fig. 1C), 25 and 50 ng/L of E₂ (Fig. 1A), 250 and 500 µg/L of BPA (Fig. 1B), E_2 +BPA ($\sum 1.25TU$ and $\sum 1.5TU$) (Fig. 1D), E_2 +NP $(\sum 1.25TU, \sum 1.5TU \text{ and } \sum 1.75TU)$ (Fig. 1E) and BPA + NP $(\sum 1.25TU, \sum 1.25TU)$ $\overline{\sum}$ 1.5TU and $\overline{\sum}$ 1.75TU) (Fig. 1F) treated groups for 15 days (P < 0.01); the vtg1 mRNA levels increased significantly in the E_2+NP ($\sum 1.75TU$) (Fig. 1E) and BPA + NP ($\sum 1.75TU$) (Fig. 1F) treated groups for 12 days. However, there was no significant difference between other treated groups and control group (P > 0.05). The estrogenic potentials of E_2 +BPA ($\sum 1.75TU$) (Fig. 1D) was the most potent, which were about 1.27, 3.14, 1.93 and 3.33 times more potent than that of 50 ng/L of E_2 (Fig. 1A), 500 µg/L of BPA (Fig. 1B), $E_2 + NP(\sum 1.75TU)$ (Fig. 1E) and BPA + NP($\sum 1.75TU$) (Fig. 1F) for 15 days, whereas no significant estrogenic potential appeared after exposed to the various concentrations of NP (Fig. 1C).

3.2. EC₅₀ values of vtg1 mRNA expressions

The relationship between the EC₅₀ values of relative *vtg1* mRNA level and the nominal concentrations (or sums of toxic units obtained from binary mixture) of E₂, BPA, E₂+BPA, E₂+NP and BPA + NP was ascertained using SPSS (Inc, 1994) for Logistic probit analysis. The EC₅₀ was observed for zebrafish exposed to E₂, BPA, E₂+BPA, E₂+NP and BPA + NP at the 3, 6, 9, 12 and 15 days (Table 2). The EC₅₀ values of individual and binary exposure were found to increase at the first 3 and 6 days and then decrease at the 6, 9, 12 and 15 days. In the NP treated groups no EC₅₀ values were observed, as it did not reach the LOEC of 100 µg/L NP for *vtg1* mRNA induction

(Jin et al., 2009). Moreover, the combined toxic effects of binary mixtures of E_2 , BPA, and NP on the biomarker vtg1 gene of zebrafish were evaluated using TU approach. The EC_{50} values for E_2 +BPA, E_2 +NP and BPA + NP for 3, 6, 9, 12 and 15 days were calculated as 95% confidence interval covers 1.0 (Table 2), respectively.

3.3. Development of RLE model based on the toxicity endpoints of EC_{50} values

Based on the EC_{50} values of vtg1 mRNA levels exposed to different concentrations of E_2 , BPA, NP and their binary mixture for 3, 6, 9, 12 and 15 days, the effect of exposure times can be further investigated by using the RLE model. Consistent with previous studies, LC_{50} (lethal concentration) and EC_{50} (effect concentration) are frequently used in various tissues as toxicity endpoints, which is related to nominal concentration and logKow (octanol partition coefficient) (Verma et al., 2014). And due to the ILC_{50} is related to the LC_{50} (Verma et al., 2015), the relationship could be extended from the IEC_{50} to the EC_{50} . Consequently, the relationship could be extended from the LC_{50} to the EC_{50} in the RLE model. Based on this extension a RLE model for estrogenic effect was proposed and may be described by the equation given below:

$$EC_{50} = -\operatorname{aln} ET_{50} + b \tag{4}$$

where EC_{50} is the effective concentration, ET_{50} is the exposure time, NLT_{50} is the normal life expectancy of the organism, and a and b are constants as previously defined. The plots of the natural log of exposure time ($\ln ET_{50}$) against EC_{50} in this study based on Equation (4) are shown in Fig. 2. Since the relationship between EC_{50} and the exposure time obtained in this study is linear, the Equation (3) is not extended by analogy.

Fig. 2 indicates that relationship between toxicity endpoints (denote by EC_{50}) of E_2 , BPA, E_2 +BPA, E_2 +NP and BPA + NP and exposure time were obtained. All plots irrespective of endocrine disrupters type had negative slopes indicating that toxicity was related to lnET₅₀ and EC₅₀ as toxicity endpoints decreased consistently in all cases. The characteristics of all plots of exposure data and calculated normal life expectancy (NLT) with reported NLT obtained from published papers with zebrafish are listed in Table 3. The slope of E₂, BPA, E₂+BPA, E₂+NP and BPA + NP is -4.43E-9, -4.18E-5, -0.2300, -0.2217 and -0.2104, respectively. The intercept of E₂, BPA, E₂+BPA, E₂+NP and BPA + NP is 3.12E-8, 2.94E-4, 1.6358, 1.5783 and 1.4990, respectively. With data sets of E₂, BPA, E₂+BPA, E_2 +NP and BPA + NP, the correlation coefficient was greater than 0.96. The reported NLT ranging from 930 to 1980 days and the calculated NLT ranging from 1123 to 1240 days in Table 3. The calculated NLT was within the reported NLT of the literature.

$3.010^{-5} \underbrace{1.500^{-5}}_{\text{L}_{2}} \underbrace{1.500^$

Fig. 2. Plots of EC₅₀ versus $lnET_{50}$ for zebrafish with linear regression lines for E₂, BPA, E₂+BPA, E₂+NP and BPA + NP data sets. Normal life expectancy (NLT) is indicated, where red dots on x-axis represents reported NLT range of zebrafish. The green, magenta, violet, orange and olive lines are displayed as regression lines for E₂, BPA, E₂+NP and BPA + NP, respectively. The horizontal and vertical dash lines indicate specific EC₅₀ values and corresponding ET₅₀ values.

3.4. Molecular docking analysis of zebrafish ER α and ligands

Zebrafish ERa sequence was selected to construct the homology model, and to analyze the interaction potential between the zebrafish ER_α-LBD and E₂, BPA and NP, and to further identify the key amino acids in the zebrafish $ER\alpha$ that interact with E_2 , BPA and NP. It can be seen from Fig. 3 that both E₂ and BPA could bind to the key amino acids through hydrogen bonds. They all could bind to the zebrafish ERα via one and more hydrogen bonds and the hydroxyl group on the phenol ring acts as the hydrogen bond donor in the zebrafish ER_α-LBD. The binding pocket of E₂, BPA and NP in the ERa-LBD was a hydrophobic cavity composed of Leu79, Met83, Phe99, Ile119 and others. Table 4 lists the intermolecular interactions, the key amino acids for stabilized combination and the interaction potential of E2, BPA and NP binding for ERa-LBD. The interaction potential of E_2 (Binding energy: -10.1 kcal/mol) for the zebrafish ERa-LBD was the most potent (stable), followed by BPA (-8.0 kcal/mol) and NP (-6.8 kcal/mol) (Table 4).

Shown in Fig. 3A is a combination of E₂ and the key amino acids of Phe99, Ile119 and others, which were formed by Van der Waals and hydrophobic stacking interactions. The phenolic hydroxyl of E₂ on one side formed hydrogen-bondeds to stabilized the combination with the side chain atoms of the key amino acids of Arg89 and Glu48 (polar residue). Shown in Fig. 3B is a combination of BPA and the key amino acids of Leu79, Met83 and others, which were formed by Van der Waals and hydrophobic stacking interactions. The docking simulation revealed that BPA adopted a binding mode with one phenol rings to the key amino acids of Arg89 on one side. which has a Pi-sigma interaction to stabilized combination. Also, the phenolic hydroxyl of BPA on one side was hydrogen-bonded to the N atom of Ala45 (non-polar residue) skeleton and the O atom of Leu41 (non-polar residue) skeleton, in order to stabilized the combination. Shown in Fig. 3C is a combination of NP and the key amino acids of which were formed by Van der Waals and hydrophobic stacking interactions. However, it shows no stronger hydrogen-bonded and Pi-sigma interactions and its binding capacity in three ligands was the weakest.

4. Discussions

4.1. Effect difference of vtg1 mRNA induction

Relative mRNA expressions of *vtg1* increased significantly for treated groups for E2 and BPA and binary mixtures. This could be explained by the fact that the concentration levels were kept at above the LOEC of $\leq 0.1 \ \mu g/L \ E_2$ for *vtg1* mRNA induction (Jin et al., 2009) and the LOEC of 500 $\mu g/L$ BPA for *vtg1* mRNA transcription (Herrera and Jagadeeswaran, 2004) in zebrafish. For NP treated groups with 5 $\mu g/L$ of NP for 15 days and other NP treated groups no significant transcriptional changes (Fig. 1C) (P > 0.05) were observed, and this could be ascribed to the fact that its concentration didn't reached the LOEC of 100 $\mu g/L$ NP for *vtg1* mRNA induction (Jin et al., 2009). However, there were significant induction of *vtg1* mRNA levels in treated groups when exposed to binary mixture of E₂+NP (Fig. 1E) and BPA + NP (Fig. 1F). This phenomenon may be related to the binding differences of E₂, BPA, and NP with the zebrafish ER α (see Section 4.6 below).

4.2. Interactive effects in binary mixtures based on EC₅₀ values

The EC_{50} values for all binary mixtures expressed in TU as shown in Table 1 were all slightly above the value of 1.0 and therefore the combined effect was categorized as an interactive type and hence the toxicity of the mixtures had a weak synergistic effect on the biomarker *vtg1* gene of zebrafish. The results showed that binary

Table 3

Regression analy	vsis of zebrafish toxicit	v data for Fo BPA	$F_{2}+BPA$ $F_{2}+NP$	P and $BPA + NP$	with the modified	linear RIF model
Regression anal	ysis of Lebransh toxicit	v uata ioi L), Di h	$L \to T \to $	and $DITI = 101$	with the mound	million REL mouch

Endocrine disrupters	Slope (a) ^a	Intercept (b) ^a	Regression coefficient (R ²)	Calculated NLT (d) ^b	Reported NLT (d)
E ₂	-4.43E-09	3.12E-08	0.9788	1156	930 (Herrera and Jagadeeswaran, 2004)
BPA	-4.18E-05	2.94E-04	0.9851	1123	1080 (Dede, 2017)
E ₂ +BPA	-0.2300	1.6358	0.9727	1227	1260 (Gerhard et al., 2002)
E ₂ +NP	-0.2217	1.5783	0.9695	1234	1350 (Herrera and Jagadeeswaran, 2004)
BPA + NP	-0.2104	1.4990	0.9665	1240	1980 (Keller et al., 2006)

The slope (a) and intercept (b) were obtained from the regression equations.

^a For $EC_{50} = -alnET_{50} + b$.

^b For $\ln NLT_{50} = b/a$.



Fig. 3. Predicted binding modes obtained from molecular docking analysis of E_2 (A), BPA (B) and NP (C) for zebrafish estrogen receptor α (ER α) docked in the zebrafish ER α -LBD.

mixtures of E_2 , BPA, and NP could produce an interactive effects under the low concentrations of exposure of their corresponding sums of toxic unit (Σ TU), which was consistent with the combined effect of the different xenoestrogens to result in an increase of the estrogenic effects, even when each individual exposure was present at low concentrations or below thresholds (Lv et al., 2007; Sun et al., 2009).

4.3. Ranking of estrogenic potentials

From the etrogenic effect data, the estrogenic potentials of low concentrations of BPA and NP with the natural estrogen E_2 and their

binary mixtures could be ranked as in the following order: E₂+BPA > E₂ > E₂+NP > BPA > BPA + NP >>>NP. This was in agreement with the estrogenic potential of binary mixture obtained in other studies. For example, MVLN cells were exposed to E₂ and BPA mixture in fixed ratios produced a significant response, and showed a further increased estrogenic effect above the E2 exposure (Bonefeld-Jorgensen et al., 2007); the fixed ratios of E2 and NP mixture produced a 17-fold increase in plasma VTG induction in juvenile rainbow trout, and the induction fold was higher than that of NP exposure (Thorpe et al., 2001); the vtg mRNA expression from the adult swordtail liver were exposed to the mixture of BPA and NP was more potent than a single exposure to NP (Kwak et al., 2001). Although the experimental organisms and exposure endpoints of the different studies were different, the ranking of estrogenic potential difference between E₂, BPA and NP and their mixture was similar.

4.4. Correlation between exposure period and liver injury of zebrafish

The respectively EC₅₀ values of individual and binary exposure of E₂, BPA, and NP were found to firstly increase and then decrease during the expose period at the 3, 6, 9, 12 and 15 days (Table 1), which was a whole exposure period of effect levels, rather than a specific effect level. This was consistent with in previously studies that plasma concentrations of endocrine disrupters rises first and descends later with exposure time (Mielke and Gundert-Remy, 2009). This tendency may explained as follows. Firstly, there was some water loss in zebrafish during the treatment, and some effect loss of the compounds being dissolved in water, which led the higher observed EC50 values to activated estrogen-responsive genes. Secondly, the compound continues to accumulate in the liver of zebrafish with increased exposure time, and the estrogenresponsive genes were activated with the lower EC₅₀ values. And lastly, the concentrations of endocrine disrupters and the estrogenic sensitivity observed for the zebrafish decreased because of the increased metabolism of the zebrafish hepatocytes and then the zebrafish liver injury became exacerbated (Lindholst et al., 2003). It was noteworthy that the liver toxicity could caused reduced life expectancy of zebrafish as evidenced by the explanation of EC₅₀ values trend of expressions of the zebrafish hepatic vtg1.

Table 4

Molecular modeling of ligand interaction between zebrafish estrogen receptor α (ER α) ligand-binding domains (LBDs).

Ligand	Intermolecular interactions	Key amino acids for stabilized combination	Binding energy (kcal/mol)
E ₂	hydrogen bonds	Arg89 and Glu48	-10.1
BPA	Pi-sigma interaction	Arg89	-8.0
	hydrogen bonds	the N atom of Ala45 skeleton and the O atom of Leu41 skeleton	
NP	no	no	-6.8

4.5. Application of RLE model based on the EC₅₀ values

The time-response relationships of xenoestrogens seen in the linear RLE model were more sensitive and intuitive (Fig. 2). There are two points in the RLE model, which are relatively fixed. One is the EC₅₀ for the compounds at relative short exposure time and the other one is the normal life expectancy of the zebrafish. The characteristics of these relationships are shown in Table 3, which was highly correlated with the intercept representing the EC₅₀ as extrapolated to an lnET₅₀ of zero and the slope indicates the rate of descent in the EC₅₀ per unit of natural log of exposure time. The more prolonged the exposure, the greater the toxic effect of the compounds and the lower the EC₅₀ which was consistent with the previously studies (Connell and Yu, 2008; Connell et al., 2016). However, it takes a long time for the compound to bioconcentrate into the fish after exposure, it's impossible to obtain a zero time for the RLE model (Connell et al., 2016), which was similar with respect to the explanation of EC₅₀ tendency in currently study. In addition, the use of calculated NLE as a limiting reference point is novel since it limits the toxicity endpoints of EC₅₀ to the range of corresponding exposure time, which was consistent with in previous studies based on lethal toxicity for the RLE model (Verma et al., 2013). Therefore, it also provides a new perspective that EC₅₀ value was similarly used as LC50 value as toxicity endpoint used for timeresponse analysis in the linear RLE model.

4.6. Interpretation of interactive effects based on the molecular docking

For E_2 +BPA mixtures, E_2 and BPA have similar to binding energy (Table 4), so they have the equivalent chance to bind to the zebrafish ERa. The concentrations of E₂ and BPA were much higher than those of other two mixtures (Table 2). When the concentrations of E₂+BPA were elevated, the mixtures would have more opportunities to bind to the zebrafish ERa. Thus, the combined effects of E_2 +BPA were of a synergistic type. For BPA + NP mixtures, the situation was a similar. Since the concentrations of NP in mixtures were lower (Table 2), the combined effects of E_2 +BPA are higher than that of BPA + NP (Fig. 1), and the combined effects of BPA + NP was also synergistic. For E₂+NP mixtures, two factors were affecting the binding energies. Firstly, the binding energy of E₂ was much higher than that of NP (Table 4), so E₂ can bind to the zebrafish ER α much easier than NP; and thus, E₂ may bind to more the zebrafish ER α than NP. Secondly, the estrogenic activity of E_2 was much higher than that of NP and BPA (Routledge and Sumpter, 1996), and the concentration of NP in mixtures was lower (Table 4). So the combined effects of E_2+NP was higher than that of BPA + NP mixtures, and was lower than that of E₂+BPA mixtures; and the combined effect of E₂+NP was also synergistic. This was in agreement with the order of estrogenic potential of individual and binary mixtures. These analyses were also referred to in previously studies (Zhang et al., 2011; Yao et al., 2013). Therefore, it was understandable that the synergistic of combined effects considering the similar action mechanism and similar molecular structure of the E2, BPA and NP.

5. Conclusions

This study investigated the concentration-time-structure response of the low concentrations of BPA and NP with the natural estrogen E_2 and their binary mixtures. The relative mRNA levels of vtg1 in adult male zebrafish was effectively upregulated following exposure to individual and binary mixture of E_2 , BPA and NP. Also, the binary mixtures of E_2 , BPA, and NP had a weak synergistic effect under the low concentrations exposure. With the

expression of hepatic vtg1 gene, the hepatic toxicity could be described by the reduced life expectancy model of zebrafish. The RLE model analyses can be useful to evaluate the exposure time effects of zebrafish by using EC_{50} as toxicity endpoint. The molecular docking analysis can be useful to understand interactive effects of xenoestrogens. The study method used may be applicable not only for zebrafish but also for other species of teleost fish and even other organisms.

Conflicts of interest

The authors declare no competing financial interest.

Acknowledgments

This study was financially supported by National Natural Science Foundation of China (Grant No.41676110)and NSFC-Guangdong Province Joint Key Project (U1301235).

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