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Vitellogenic responses of male Chinese loach (*Misgurnus anguillicaudatus*) exposed to the individual or binary mixtures of 17β-estradiol and nonylphenol

LÜ XueFei, ZHOU QunFang, SONG MaoYong, JIANG GuiBin† & SHAO Jing

State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Beijing 100085, China

Male Chinese loaches were exposed to 17β-estradiol (E2) and nonylphenol (NP) both singly and in combination for 42 days using semi-static waterborne exposure system. Plasma vitellogenin (Vtg) was chosen as determining endpoint. The results demonstrated that 0.5 μg/L E2 could induce the enhancement of Vtg contents in male Chinese loaches after exposure for 21 days, which showed a time-related increasing manner; NP was also estrogenic to male Chinese loach, and the vitellogenic responses showed in a time- and dose-related manner, which was less potent than that of E2. The binary mixtures of E2 and NP can significantly elicit the production of Vtg in male Chinese loaches, which was more potent than that of individual compounds, and Vtg induced in the binary mixture groups was higher than the summation of Vtg induced in the corresponding single-compound groups at the same concentration.

chemical interaction, Chinese loach, endocrine disruption, 17β-estradiol, *Misgurnus anguillicaudatus*, nonylphenol, vitellogenin

A great number of studies have focused on endocrine disrupting chemicals (EDCs), which pose an adverse effect on the endocrine system of wildlife and humans. EDCs include a variety of compounds, such as the natural and synthetic steroidal estrogens, alkylphenols, organochlorine pesticides, and phthalates[1]. Nonylphenol (NP), the degradation product of industrial nonionic surfactants (nonylphenol polyethoxylates) during sewage treatment, has been reported to be a weak estrogenic compound in various *in vitro* and *in vivo* assays[2−9].

Vitellogenin (Vtg) is an egg yolk precursor lipophosphoprotein, which is normally produced in sexually maturing females as a response to endogenous estrogens circulating in the plasma. Under natural conditions, males cannot synthesize Vtg. However, they also possess the Vtg gene, which can be expressed when exposed to estrogen or estrogen mimics. So abnormal levels of Vtg in male fish can be used as a good biomarker to demonstrate estrogenic effects of estrogens or estrogen mimics in the aquatic environment[10−13].

Chinese loaches are a kind of widespread freshwater fish in Asia. The attractive characteristics, for instance, moderate size, being easy to culture and identifying gender, make them suitable as sentinel fish in field and laboratory studies[14]. Moreover, the results of our previous study demonstrated that Chinese loach was sensitive to 17β-estradiol[15]. Although NP has been demonstrated to be estrogenic in different fish species, such as flounder (*Platichthys flesus*)[5], medaka (*Oryzias latipes*)[7], zebrafish (*Danio rerio*)[16], and rainbow trout (*Oncorhynchus mykiss*)[17], little is known about the estrogenic effect of NP on Chinese loach.

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†Corresponding author (email: gbjiang@rcees.ac.cn)
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The majority of the current studies on EDCs focused on the estrogenic effect of single compound; however, as a matter of fact, humans and wildlife live in a natural environment containing a variety of estrogenic chemicals. Therefore, risk assessment cannot be accurately made on the basis of current toxicological data of single compound. It will be more important to study the effects of mixtures than those of the individuals. A number of studies have demonstrated that significant estrogenic effects could occur in the mixture compounds at levels below individual no-observed-effect concentration[18−22]. Moreover, Rajapakse et al.[23] demonstrated that BPA could contribute to the overall mixture effect in the presence of E2 using the yeast estrogen screen (YES). However, in the presence of E2, the contribution of alkylphenols to the effects of the mixtures needs to be studied.

So, in this study, in order to investigate the estrogenic effect of NP in male Chinese loach and the contribution of NP to the effects of the binary mixture of E2 and NP, male Chinese loaches were exposed to the individuals and binary mixtures of E2 and NP for 42 days using semi-static waterborne exposure system, and plasma Vtg level was chosen as the determining endpoint.

1 Materials and methods

1.1 Apparatus and reagents

The high performance ion-exchange membrane chromatography system includes anion exchange membrane (Sartobind™ MAD15X, Germany), high flow rate HPLC pump (Allitech 426, USA) and UV detector (SPD-10A VP, Shimadzu, Japan); ultracentrifuge MR1822 (Jouan, France); microtiter plate reader (DNA Expert, TECAN, Austria).

17β-estradiol (E2, 98% purity) was obtained from Sigma (USA); 4-Nonylphenol (NP, mixture of compounds with branched sidechain) was purchased from Tokyo Kasei Kogyo (Japan); Aprotinin was purchased from Boehringer (Germany); Bovine serum albumin (BSA) was obtained from Roche (Germany); *o*-phenylenediamine (OPD) was obtained from Amresco (USA); enzyme-labeled second antibody [horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG] was obtained from the Academy of Military Medical Sciences (Beijing, China), and other chemicals were reagent-grade compounds obtained from Beijing Chemical Reagent Company (China).

1.2 Fish

Matural male Chinese loaches were used in this study. Healthy Chinese loaches were obtained from the market of Beijing, China. The mean weight and length of fish were 9.13±1.69 g and 12.61±0.70 cm, respectively. Prior to the experiments, Chinese loaches were acclimated in stainless steel tanks for at least 2 weeks, which were maintained under the natural day length and were provided with dechlorinated tap water. The controlled parameters of water qualities included pH (6.9－7.9), oxygen concentration (5－7 mg/L), and temperature (22.5－25.5℃).

1.3 Experimental design

Stock solutions of E2 and NP were dissolved in methanol, and the working solutions were diluted with ultrapure water (EASY pure LF) from the corresponding stock solutions. All the solutions were sealed and stored at 4℃.

At the beginning of the experiment, fish was randomly allocated to 12 L glass tanks with a working volume of 5 L. Two aquaria were dosed at the same concentration. Eleven exposure groups were set in the following: E2 at the nominal concentration of 0.5 μg/L; NP at nominal concentrations of 50, 200, 500, and 1000 μg/L; binary mixtures of E2 and NP at nominal concentrations of 0.5 μg/L + 50 μg/L, 0.5 μg/L + 200 μg/L, 0.5 μg/L + 500 μg/L, and 0.5 μg/L + 1000 μg/L; the solvent control and control tanks received 0.02% methanol and dechlorinated tap water, respectively. Water was changed every other day during the exposure. The fish was fed with *Oligochaetes limnodirlus* sp. twice a week. The exposure period was controlled for 42 days.

1.4 Plasma collection

Fish (n = 4 for each dose) was randomly sampled on day 7, 14, 21, 28, 35, and 42 of the whole exposure. Before sampling, fish was anesthetized with quinoline sulfate (40 mg/L), and then blood samples were taken from the caudal vein using heparinized syringes and transferred to 1.5 mL centrifuge tubes in the presence of aprotinin. After centrifugation (3000 r/min, 4℃, 30 min), plasma was collected, divided into aliquots and stored at –20℃ for Vtg analysis.

1.5 Vitellogenin determination

Plasma Vtg concentrations were determined with a competitive enzyme-linked immunosorbent assay (ELISA) described elsewhere[14]. Briefly, coating the 96-well microtiter plate with 750 ng/mL purified Vtg in Na_2CO_3-$NaHCO_3$ buffer solution (pH = 9.6), staying at room temperature for 2 h, and then transferring to 4℃ overnight; washing the plate four times with PBST buffer (0.02 mol/L phosphate buffer with 0.15 mol/L NaCl and 0.05% Tween 20, pH 7.4), and then adding 1% BSA incubated at 37℃ for 2 h; washing the plate, adding standards and samples (incubated with primary antibody solution at a final dilution of 1∶16000 in PBST overnight at 4℃) incubated at 37℃ for 2 h; washing the plate, adding enzyme-labeled second antibody [horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG at a final dilution of 1∶2000 in PBST] incubated at 37℃ for 1.5 h; washing the plate, adding the enzyme substrate solution (0.05 mol/L phosphate-citrate buffer, 0.4 mg/mL *o*-phenylenediamine, and 0.16% H_2O_2) incubated at 37℃ for 30 min; adding 2 mol/L HCl to stop the reaction and measuring the absorbance at 490 nm. The detection limit of the assay was 5.7 ng/mL, the intra- and inter-assay coefficients of variations were 6.9% and 10.4%, respectively.

1.6 Data expression and statistical analysis

All the values were expressed as mean ± SD. The ELISA data were processed by a four-parameter Boltzmann equation: $y = a + \frac{b}{1+\exp\left(\frac{x-c}{d}\right)}$, where y represents the percentage binding of sample or standard relative to analyte free wells (B_i/B_0) and x represents log dose. The effects of different treatments on Vtg concentrations were described using one-way analysis of variance (ANOVA). The level of significance was set as $P<0.05$.

2 Results and discussion

2.1 Vitellogenic responses of male Chinese loach exposed to E2 and NP separately

Vitellogenic responses of male Chinese loach exposed to E2 at the nominal concentrations of 0.5 μg/L and NP at the nominal concentrations of 50, 200, 500, and 1000 μg/L for 42 days are shown in Figure 1. The results indicated that the average Vtg levels of all the exposed groups ranged from not detected (N.D.) to 243.27±27.30 μg/mL (1000 μg/L NP for 42 days). Vtg levels in the control and solvent control groups ranged from not detected (N.D.) to 26.94±6.87 μg/mL. Relative high levels Vtg can be detected in the solvent control group, which might be caused by the contamination of estrogenic compounds in the exposure experiment, and the reasons remain unsolved.

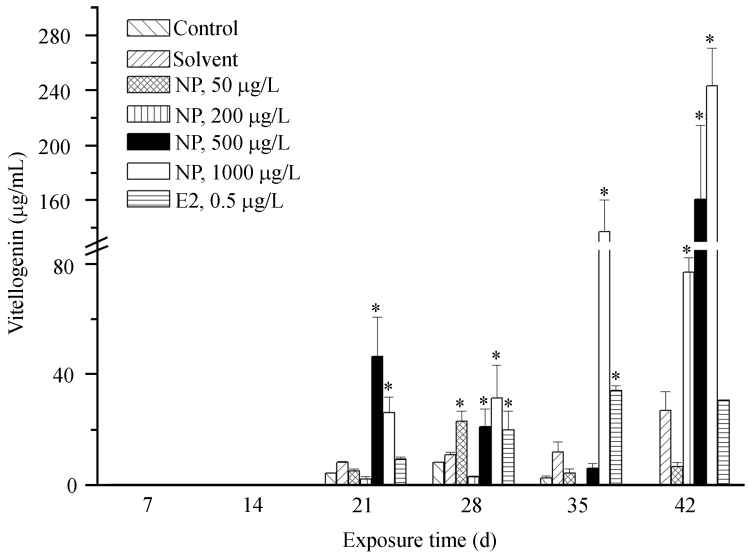

Figure 1 Vitellogenic responses of male Chinese loach exposed to E2 at the nominal concentration of 0.5 μg/L and NP at the nominal concentrations of 50, 200, 500, and 1000 μg/L for 42 days. Values are mean ± SD. * denotes significant differences from the control groups at $P<0.05$.

For E2 exposure group, it could be found that Vtg levels showed an increasing pattern from 9.29±0.71 μg/mL on day 21, to 19.98±6.85 μg/mL on day 28, to 34.15±1.71 μg/mL on day 35, and to 30.59±0.24 μg/mL on day 42. Vtg production induced by E2 in other fish species has also been reported. For example, exposure to 20 ng/L E2 for 21 days can significantly induce the production of Vtg in adult male zebrafish (*Danio rerio*) and juvenile rainbow trout (*Oncorhynchus mykiss*) ($P < 0.001$)[16]; Vtg contents in male fathead minnow (*Pimephales promelas*) was significantly enhanced after exposure to 30 ng/L E2 for 21 days ($P<0.01$)[24].

NP was estrogenic to male Chinese loach, and Vtg induction showed a time- and dose-dependent manner, which was less potent than that of E2. During the whole exposure period (42 days), 50 μg/L NP did not significantly induce the production of Vtg in male Chinese loach except for day 28. Compared with the control and solvent control groups, Vtg levels were significantly induced in the samples exposed to both 200 and 500 μg/L NP for 42 days ($P<0.05$), which were 77.13±5.26 μg/mL and 161.05±53.53 μg/mL, respectively. 1000 μg/L NP can significantly induce the production of Vtg within a 21-day exposure with an average value of 26.07±5.63 μg/mL ($P<0.05$), and Vtg levels in this treatment increased from 31.47±11.92 μg/mL on day 28 to 243.27±27.30 μg/mL on day 42. Numerous studies have demonstrated the estrogenic effect of NP using different fish species as experimental animal. For example, 5 μg/L NP did not elicit significant Vtg induction in sexually matural male carp (*Cyprinus carpio*)[25]. Pedersen et al.[17] found that 150 μg/L tech-NP can create a significant increase of Vtg at the level of 1248±382 μg/mL in juvenile rainbow trout (*Oncorhynchus mykiss*) after a 9-day water exposure. And juvenile rainbow trout was demonstrated to possess the significantly elevated Vtg levels after exposure to 1 μg/L NP for 1 year[26].

Compared with the results in other fish species, it can be concluded that male Chinese loach was less sensitive, which might be caused by many factors. Firstly, the reproductive stages of fish species, for example, juveniles were more sensitive than adults; secondly, the exposure conditions, for example, flow-through exposure system can provide more stable exposure concentrations than semi-static exposure system; thirdly, the purity of the exposure chemicals, because it has been demonstrated that the estrogenic activity of branched and linear alkylphenols showed large discrepancy[17]; in addition, fish species itself might be an important factor in its sensitivity to endocrine disrupting chemicals.

2.2 Vtg induction in male Chinese loach exposed to the binary mixture of E2 and NP

Figure 2 shows the vitellogenic responses of male Chinese loaches exposed to the binary mixtures of E2 and

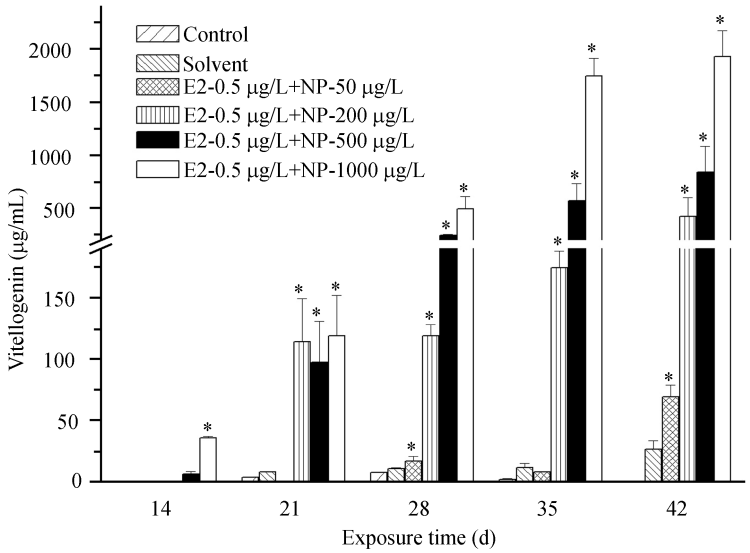

Figure 2 Vitellogenic responses of male Chinese loach exposed to the binary mixtures of E2 and NP at nominal concentrations of 0.5 μg/L + 50 μg/L, 0.5 μg/L + 200 μg/L, 0.5 μg/L + 500 μg/L and 0.5 μg/L + 1000 μg/L for 42 days. Values are mean ± S.D. * denotes significant differences from the control groups at $P<0.05$.

NP at the nominal concentrations of 0.5 μg/L + 50 μg/L, 0.5 μg/L + 200 μg/L, 0.5 μg/L + 500 μg/L, and 0.5 μg/L + 1000 μg/L for 42 days. It can be found that the estrogenic effects of the binary mixtures of E2 and NP showed an obvious time- and dose-dependent manner during a 42-day exposure. Vtg contents of the exposed groups ranged from not detected (N.D.) to 1.93±0.25 mg/mL (0.5 μg/L E2 + 1000 μg/L NP for 42 days).

For the binary mixture exposure group of 0.5 μg/L E2+50 μg/L NP, a 21-day exposure did not induce the production of Vtg, and Vtg levels increased from 17.4±3.68 μg/mL on day 28 to 69.48±9.70 μg/mL on day 42, however, those levels of Vtg did not show significant difference compared with those of the control groups ($P > 0.05$); for the male Chinese loaches exposed to 0.5 μg/L E2 + 200 μg/L NP, no Vtg occurred after a 14-day exposure. However, Vtg was significantly elevated from 114.13±35.06 μg/mL on day 21 to 427.06± 175.33 μg/mL on day 42 ($P<0.05$), which were all higher than those in fish exposed to 200 μg/L NP and 0.5 μg/L E2 alone; Vtg contents in male Chinese loaches exposed to 0.5 μg/L E2 + 500 μg/L NP were significantly elevated from day 21 with a mean concentration of 97.44±33.64 μg/mL ($P<0.05$), and the maximal Vtg levels in this treatment were obtained on day 42 with an average value of 840.80±246.60 μg/mL, which was more than 5-fold that produced in 500 μg/L NP for 42 days. Although Vtg cannot be detected in the group of 0.5 μg/L E2+1000 μg/L NP within 7 days, 36.2±1.23 μg/mL Vtg was significantly induced after a 14-day exposure ($P < 0.05$), and the maximal Vtg level of 1.93 ± 0.25 mg/mL was obtained after exposure for 42 days.

Vtg contents in the co-exposed groups all exceeded those of the two compounds present individually. Figure 3 shows the comparison between Vtg levels in the E2+NP groups and the summation of Vtg levels in the individual E2 and NP groups after exposure for 42 days. It can be clearly observed that after exposure for 42 days, except for 0.5 μg/L E2 + 50 μg/L NP, Vtg contents in the other co-exposed groups (0.5 μg/L E2 + 200 μg/L NP, 0.5 μg/L E2 + 500 μg/L NP, and 0.5 μg/L E2 + 1000 μg/L NP) were all significantly higher than the summation of Vtg in the corresponding single-compound groups at the same concentration.

Thorpe et al.[27] showed that the binary mixture of 4.9 ng/L E2 and 3.3 μg/L NP could induce the production of

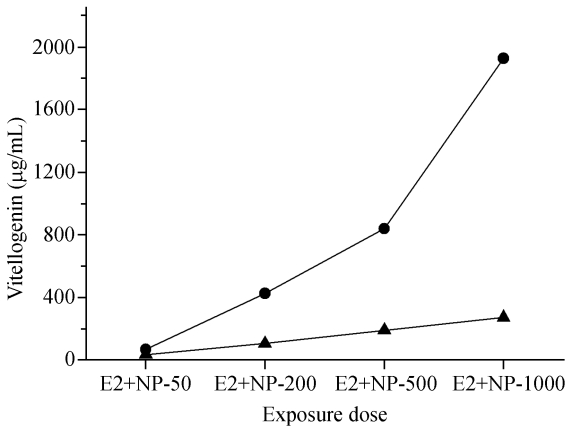

Figure 3 Comparison between Vtg levels in the E2+NP groups (0.5 μg/L + 50 μg/L, 0.5 μg/L + 200 μg/L, 0.5 μg/L + 500 μg/L, and 0.5 μg/L + 1000 μg/L) (●) and the summation of Vtg levels in the individual E2 and NP groups (▲) after exposure for 42 days.

8720±4370 ng/mL Vtg in juvenile rainbow trout (*Oncorhynchus mykiss*), which was 18-fold the Vtg contents induced by the corresponding single compounds at the same concentration. Moreover, concentration addition (CA) model predicted that NP acted in an additive manner with E2. The results of Brian et al.[22] demonstrated that a mixture of estrogenic compounds (E2, EE2, NP, OP and BPA) act in an additive manner in male fathead minnows, which can be predicted accurately using CA model.

The present study only investigated the vitellogenic responses of male Chinese loaches to E2, NP and the binary mixtures of E2 and NP, and from the information obtained herein, it can be definitely observed that estrogenic effects of the binary mixture of E2 and NP were more potent that that of the individual compound, even higher than the summation of Vtg in the corresponding single-compound groups at the same concentration. However, it cannot be concluded whether the effects of E2 and NP were additive or synergistic, which needs to be further studied in the following experiments.

3 Conclusion

The estrogenic effects of E2, NP and E2+NP were investigated in the present study using male Chinese loach as experimental animal and Vtg as determining endpoint. The results demonstrated that NP was estrogenic to male Chinese loaches, which was less potent than that of E2; the estrogenic effect of the binary mixture of E2 and NP was more potent that those of individual compounds, and Vtg contents induced in the binary mixtures were all significantly higher than the summation of Vtg in the

corresponding single-compound groups at the same concentration. The results indicated that in the presence of strong estrogenic compound (E2), weak estrogenic compound (NP) could still contribute to the overall effects of the mixtures, which highlighted the importance of taking account of the combination effects of the mixtures of endocrine disrupting chemicals during environment hazard and risk assessment.

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