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Combination Effect of Bisphenol A and Nonylphenol to Japanese Medaka (*Oryzias latipes*)

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일본산 송사리 (*Oryzias latipes*)에 대한 Bisphenol A와 Nonylphenol의 혼합효과

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요 약

생활하수, 공장폐수, 농경유출수에 의해 수생태계로 유입된 다양한 화학물질들은 수서곤충이나 어류와 같은 수생생물에게 나쁜 영향을 주곤 한다. 비스페놀A와 노닐페놀을 포함하는 많은 화학물질들이 내분비계 장애물질(EDCs)로 의심되고 있고, 그들은 환경속에서 서로 다른 혼합형태로 공존하기도 한다. 따라서 비스페놀A와 노닐페놀의 혼합물이 독성과 생식학적 반응에 미치는 영향을 살펴보기 위해 일본산 송사리의 수정란 치사율, 부화율 및 부화시간, 치어의 성장율 및 비텔로제닌 농도 등이 측정되었다. 수정된 지 24시간 이내의 수정란을 대조군, 양성대조군(17β-estradiol), 그리고 서로 다른 농도의 비스페놀A와 노닐페놀의 혼합물에 부화 후 60일까지 유수식 조건하에 노출시켰다. 수정란~치자어 단계에서는 대조군과 비교하여 실험군의 치사율 및 부화율, 부화시간에 차이가 나타나지 않았으며, 부화 후 60일간의 노출 후 성장(길이, 무게)에 있어서도 비록 양성대조군에서 낮은 성장상태를 보였지만 다른 혼합물의 실험군들과는 차이를 보이지 않았다. 한편 체내 비텔로제닌 농도는 혼합물의 농도증가에 따라 증가하였으며 수컷의 경우 최저농도의 혼합물(Treatment A)을 제외한 실험군에서 농도증가에 따라 증가하였다. 반면 양성대조군의 경우 수컷이 발견되지 않았고 암컷 체내의 비텔로제닌 농도는 최고농도의 혼합물(Treatment D) 실험군과 비슷한 경향을 보였다. 위 실험을 통해 각각의 내분비계 장애물질이 개별적으론 생식발달 및 비텔로제닌 유도에 무영향농도(NOEC)라 하더라도 혼합된 경우 영향이 나타날 수 있다는 것을 보여주었으며, 이는 수환경 내 다양한 화학물들의 혼합효과(combination effect)가 생태위해성평가를 좀더 면밀하게 하기 위해서 주의 깊게 고려되어야 한다고 제안한다.

Key words : bisphenol A, nonylphenol, endocrine disrupting chemicals (EDCs), medaka

INTRODUCTION

Concern over the potential impact of natural estrogen hormones and chemicals mimicking the effects

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of hormones on aquatic organism has heightened in recent years after demonstration that these chemicals can adversely affect sexual development (Sumpter and Jobling, 1995; Shioda and Wakabayashi, 2000; Seki *et al.*, 2003). Especially, a number of studies have reported elevated vitellogenin and intersex gonad of fish in polluted streams receiving effluents from sewage/wastewater treatment plants (Purdom *et al.*, 1994; Harris *et al.*, 1997; Jobling *et al.*, 1998). The cause of these estrogenic responses in wild fish is due to various natural estrogen such as estrone and 17β-estradiol, and artificial chemicals including ethinyl-estradiol (Sohoni *et al.*, 2001; Segner *et al.*, 2003; Seki *et al.*, 2003).

Although numerous chemicals are present in the aquatic environment, most studies have been reported on assessment of single chemical, and the assay tool for the effect of chemical mixture was not developed yet (Yokota *et al.*, 2000; Länge *et al.*, 2001; Kang *et al.*, 2002a). We found there was an urgent need to assess this potential problem and to develop new method for effect of mixtures of endocrine disrupting chemicals (Heppell *et al.*, 1995; Panter *et al.*, 1998; Fenner-Crisp *et al.*, 2000; Huet, 2000; Ryu, 2002).

According to the research (*Chen et al.*, 2007), bisphenol A (BPA) and nonylphenol (NP), synthetic alkylphenols with relatively weaker estrogenic activity, are highly present in U.S. rivers surveyed. However, mixtures of these weak xenoestrogens in the aquatic environment may result in estrogenic effects even though they are present below NOEC individually. In this study, we tested to well-known EDCs, BPA and NP, and focused to observe any combination effect on Japanese medaka (*Oryzias latipes*). Embryos were exposed to various nominal concentrations of BPA+ NP under continuous flow-through condition up to 60-days post-hatch and the potential effects such as mortality and hatching rate, time to hatch, growth and estrogenic response (vitellogenin induction) were observed during the exposure period (Benoit *et al.*, 1982; Kristensen, 1991; OECD, 1998; Kang *et al.*, 2002b; Yeom *et al.*, 2005).

MATERIALS AND METHODS

1. Test chemicals and fish

17β-estradiol (Sigma, contains 97% purity) was used as a positive control, and bisphenol A (Sigma, 99% purity) and nonylphenol (Aldrich, mixture of isomers) were used as estrogenic chemicals. Stock solutions of three chemicals were prepared by dissolving in acetone (< 100 μL/L), and then they were diluted with dechlorinated tap water to make the nominal treatment concentrations as follows:

Treatment A-mixture of BPA (1.2 μg/L)
and NP (1.0 μg/L)
Treatment B-mixture of BPA (80 μg/L)
and NP (6 μg/L)
Treatment C-mixture of BPA (400 μg/L)
and NP (12 μg/L)
Treatment D-mixture of BPA (2,000 μg/L)
and NP (24 μg/L)
Positive control-17β-estradiol (0.5 μg/L)

The concentrations were selected based on the previous studies and the maximum concentration can possibly occur in our environment (Yokota *et al.*, 2000; Kang *et al.*, 2002a, b; Seki *et al.*, 2003).

The fish used in this study were Japanese medaka (*Oryzia latipes*), cultured in the Korea Institute of Toxicology (Daejeon, Korea). The fish is one of well-known test species recommended for screening and testing of endocrine disrupting chemicals (U.S. EPA 1998; ECETOC 1999; OECD 1999). We chose adult medaka, approximately six months post-hatch, and placed 20 fish (5 males and 15 females) in each 40-liter mating aquarium. Eggs spawned from each female were removed until a day before the test started, and then the testing eggs fertilized within 24 hours were carefully collected in a following day. After examination of egg condition, normal fertilized eggs (embryos) were used for the exposure test.

2. Exposure design

The exposure system was continuous flow-through

followed based on recommended methods in the OECD test guideline 210 and 212. The 60 embryos in each treatment were randomly separated into three groups of 20 in each egg vessel, floating and moving up and down in the 1.7-liter test chamber. Flow rate of the test solution was 12 chamber volumes per day, the photoperiod was 16 : 8-hr light : dark, and water temperature was maintained at 24±1°C. Embryonic development was observed daily under dissecting microscope until hatched, and dead embryos were removed. Once embryos were hatched, they were carefully transferred to the test chamber. The hatched larvae were fed an adequate amount of Artemia (hatched within 24 hours) in the morning and Tetramin™ in the afternoon. Daily observation was performed to examine mortality and abnormal behavior and appearance until 60-day post-hatch, and dead fish, residual food, and feces were removed as soon as possible. The water temperature, dissolved oxygen concentration, and pH of each test chamber were monitored once weekly, these conditions were maintained to follow the requirement of test guideline. After 60-day post-hatch, all surviving fish were taken out from the chambers, and their sex was determined by observation of external secondary sex characteristics (shape of the dorsal and anal fins). They were blotted on filter paper, weighed (total weight), and measured (total length), and some of them in each treatment were randomly selected for vitellogenin analysis.

3. Measurement of VTG concentration

Whole body of selected fish were individually homogenized in 250 μL of ELISA assay buffer and centrifuged at 8,000 rpm for 10 min, and the supernatants were collected and frozen at −80°C for later ELISA analysis. Vitellogenin concentrations were measured with a medaka VTG enzyme-linked immunosorbent assay kit (EnBioTec Lab., Japan).

4. Statistical analysis

Data were reported as mean±standard error, and homogenized vitellogenin data were log-transformed. All statistical analyses were performed with the SigmaStat software (Version 2.03, SPSS Inc.). When normality and equal variance tests were passed, the data were subjected to one way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. On the other hand, the nonparametric Kruskal-Wallis test, followed by Dunn's method in multiple comparison procedure, was used when either normality or equal variance tests was failed. In all cases, differences were considered as significant when $P \leq 0.05$.

RESULTS AND DISCUSSION

1. Effect on embryo

Some dead embryos were observed randomly, and cumulative mortalities at embryo stage were in the range of 5.9 to 13.0% in all treatment groups. In the BPA (2000)+NP (24) μg/L treatment group (D), the mortality were decreased substantially, but no statistically (P=0.815) significant differences observed among treatment groups, positive control and control (Table 1).

Hatching rate and time to hatch of embryo exposed to mixture of BPA and NP showed no adverse effects (P=0.710). The combination of the highest concentration (D) in both chemicals had somehow the mini-

Table 1. Effects of exposure to combination of bisphenol A and nonylphenol on mortality, hatchability, and time to hatch of fertilized eggs of Japanese medaka (*Oryzia latipes*)

Treatment (μg/L)	Mortality (%)	Hatching rate (%)	Time to hatch (day)
Control	11.6±1.4	84.1±3.8	8.7±0.2
BPA (1.2)+NP (1)	11.6±2.9	87.0±2.5	8.2±0.2
BPA (80)+NP (6)	13.0±5.0	82.6±6.7	8.0±0.1
BPA (400)+NP (12)	13.0±5.0	87.0±5.0	8.3±0.1
BPA (2,000)+NP (24)	5.9±1.4	92.6±2.8	8.5±0.3
E_2 (0.5)	10.1±5.8	88.4±5.2	8.9±0.2
P value	0.815	0.710	0.083

Data expressed as mean±standard error (n=3)

Table 2. Effect of exposure to combination of bisphenol A and nonylphenol on growth of medaka (*Oryzia latipes*) at 60-day post-hatch

Treatment (μg/L)	Total length (mm)	Total weight (mg)
Control	21.0±0.5	87.7±6.2
BPA (1.2)+NP (1)	20.8±0.4	81.5±4.5
BPA (80)+NP (6)	21.2±0.4	84.8±4.8
BPA (400)+NP (12)	21.1±0.3	80.1±3.6
BPA (2,000)+NP (24)	20.1±0.3	69.4±2.7
E_2 (0.5)	15.9±0.5*	42.3±4.0*
P value	≤0.001	≤0.001

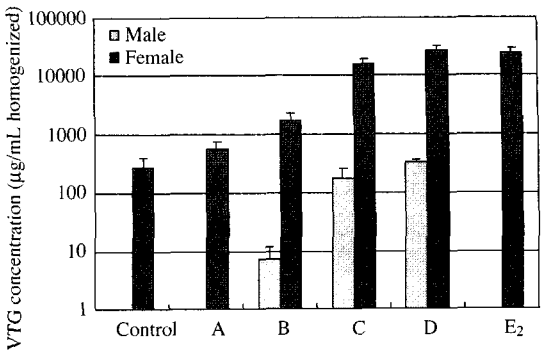

Fig. 1. The effects of different mixture concentrations of bisphenol A and nonylphenol on homogenized vitellogenin levels in medaka (*Oryzia latipes*) exposed for 60-day post-hatch.

mum mortality and the maximum hatchability, and the research (Yokota *et al.*, 2000) had similar result with 1,829 μg/L BPA. However, the hatching rate of all treatment groups and positive control and control exceeded 82 %, and the time to hatch was about 8～9 days in all groups (Table 1).

During the exposure period, embryological abnormalities such as irregular heartbeat and delayed eye formation were observed in some treatments, and these embryos died eventually in most cases. However, there was no evidence of a dose response in abnormal behavior and appearance versus exposure concentration.

2. Effect on juvenile

Compared to the control, total length (about 25%) and total weight (about 50%) of fish in the positive control decreased significantly at 60-day post-hatch ($P \leq 0.001$, Kruskal-Wallis ANOVA on ranks followed by Dunn's multiple comparison test). Even though less growth in the combination of the highest concentrations (D) was found, there were no significant differences observed in either total length or total weight of fish between the control and treatment groups (Table 2).

3. Whole body VTG concentrations

After 60-day post-hatch, vitellogenin levels in homogenized whole-body of medaka were measured in control, positive control, and treatment groups. The VTG level of female in binary combination treatment groups showed a concentration-dependent increase, and the mean values in the combination of the highest concentration (D) and the positive control were significantly increased compared with the control (P=0.001) (Fig. 1). Exposure to combination treatment groups induced the homogenized VTG concentrations of male except for those in the combination of the lowest concentration (A), and the VTG level of male in the D group differed significantly (P =0.009) from those of the control. Furthermore, the induced VTG concentrations of male fish in the D treatment group were little higher than those of females in the control. There was no male medaka in E_2 treatment, and the VTG level of female was similar to those of females in the D group.

Hatchability, time to hatch, and embryo mortality were not affected by exposure to combinations of BPA and NP. According to other studies (Yokota *et al.*, 2000; Länge *et al.*, 2001; Seki *et al.*, 2003), the hatching success of the control embryos was greater than 90%, and even 100% in high concentration of 4-nonylphenol (44.7 μg/L) or bisphenol A (1,820 μg/L). The time to hatch was about 9 to 10 days in every treatment. Compared to these results, we observed little low hatchability and less time to hatch in this study. However, these conditions suited with requirements for the OECD Test Guideline 210 and 212 (hatchability > 80%, hatching time 8～11 days).

Statistical analysis of the total length and total weight data at 60-day post-hatch indicated that no significant differences were observed in exposure to combinations of BPA and NP. The research (Kang *et al.*, 2002b) indicated that no reduction in growth was found in adult medaka exposed to BPA (up to 3,120 μg/L of measured concentration) for three weeks. However, other studies (Yokota *et al.*, 2000; Seki *et al.*, 2003) reported that both mean total length and total weight of the fish at 60-day post-hatch decreased significantly at 1,820 μg/L BPA and 44.7 μg/L 4-NP. In this study, we also found growth reduction in the combination of the highest concentrations (D). When running statistics (Kruskal-Wallis ANOVA on ranks followed by Dunn's multiple comparison test) with data except for the positive control, the fish in the highest treatment had significant reduction in both total length (P=0.030) and total weight (P=0.028). The result showed similar LOEC in the combination of both chemicals compared to LOEC of individual exposure in other studies (Yokota *et al.*, 2000; Kang *et al.*, 2002a, b; Seki *et al.*, 2003).

The phenotypic sex can be determined by sex-specific fin characters (shape of the dorsal and anal fins). In this study, we observed that feminized appearance of secondary sex characteristics. Most fish in the treatment group (D) and all fish in the positive control at 60-day post-hatch showed female secondary sex characteristics on anal fin. These findings indicated that the feminization of the secondary sex characteristics probably had been caused by the estrogenic activity of bisphenol A and nonylphenol in male fish. It has been widely accepted that estrogenic chemicals promote the expression of female secondary sexual characteristics in fish (Gray and Metcalfe 1997; Gronen *et al.*, 1999; Knörr and Braunbeck 2002). In medaka, the research (Kang *et al.*, 2002a, b) reported that various estrogens affect the formation of secondary sex characteristics on the anal fin in male medaka. Other studies indicated feminization with exposure of bisphenol A or 4-nonylphenol, and sex ratio (male : female) at ≥355 μg/L BPA and ≥23.5 μg/L 4-NP were significantly skewed toward female (Yokota *et al.*, 2000; Seki *et al.*, 2003).

Vitellogenin is normally synthesized in sexually maturing females, male fish cannot produce vitellogenin. However, they can be induced to synthesize VTG when exposed to estrogenic compounds. Detection of VTG in male fish is a simple and sensitive biomarker for endocrine disrupting chemicals (EDCs) with estrogenic effects (Sumpter & Jobling, 1995). Measurement of VTG has become an accepted routine screening test for estrogenic and anti-androgenic effects of EDCs in fish. Therefore, the present study was carried out to assess the VTG by mixture of BPA and NP. The results demonstrated that VTG in male fish at B, C, and D treatment group were induced dose-dependent manner, however VTG at a lowest treatment group (A) was not induced.

The research (Kang *et al.*, 2002b) reported that BPA concentration at 837 and 1,720 μg/L did not induce VTG synthesis, and 3,120 μg/L BPA for three weeks induced the production of hepatic VTG in adult medaka. The research (Van den Belt *et al.*, 2003) found that 1,000 μg/L BPA significantly increased plasma VTG in male zebrafish and juvenile rainbow trout, and both species exposed to 200 μg/L BPA concentration did not show induced VTG. The results (Kang *et al.*, 2003) indicated that 24.8 μg/L NP concentration did not induce VTG synthesis but significantly increased in ≥50.9 μg/L NP. From the results in this study, it can be found that vitellogenic responses of mixtures of BPA and NP were more potent than those produced in each compound tested alone. The research (Kwak *et al.*, 2001) has been reported that exposure of juvenile swordtail fish to a mixture of NP and BPA more strongly induced hepatic VTG than did either chemical alone.

In conclusion, the results of present study indicated that weak xenoestrogens are able to contribute to the overall mixture effects at low concentrations. Therefore, we should take account of the combination effects of endocrine disrupting chemicals, which will not lead to the underestimation of potential hazard during environmental hazard and risk assessment.

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