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

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INTRODUCTION

Concern over the potential impact of natural estrogen hormones and chemicals mimicking the effects of estrogen have been growing. Estrogens and chemicals that mimic the estrogenic effects (EDCs) are found in various environmental media such as wastewater, industrial effluents, and agricultural runoff. Bisphenol A and nonylphenol are known to be endocrine-disrupting chemicals (EDCs) that can cause reproductive and developmental problems in aquatic organisms.

Therefore, the study aimed to investigate the effects of the mixture of Bisphenol A and Nonylphenol on the growth, survival, and developmental parameters of Japanese Medaka (Oryzias latipes). The experiment was conducted by exposing the medaka embryos to different concentrations of the mixture for 24 hours. The results showed that the survival and hatching rates were not significantly different between the control group and the experimental groups. However, the growth of the medaka was slightly lower in the experimental groups compared to the control group.

In conclusion, the study highlights the importance of considering the combined effects of multiple chemicals in aquatic ecosystems, as the individual effects of chemicals may not always reflect their impact when combined.
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 (Oryzias 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 ≤ 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 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>
<thead>
<tr>
<th>Treatment (µg/L)</th>
<th>Mortality (%)</th>
<th>Hatching rate (%)</th>
<th>Time to hatch (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.6 ± 1.4</td>
<td>84.1 ± 3.8</td>
<td>8.7 ± 0.2</td>
</tr>
<tr>
<td>BPA (1.2) + NP (1)</td>
<td>11.6 ± 2.9</td>
<td>87.0 ± 2.5</td>
<td>8.2 ± 0.2</td>
</tr>
<tr>
<td>BPA (80) + NP (6)</td>
<td>13.0 ± 5.0</td>
<td>82.6 ± 6.7</td>
<td>8.0 ± 0.1</td>
</tr>
<tr>
<td>BPA (400) + NP (12)</td>
<td>13.0 ± 5.0</td>
<td>87.0 ± 5.0</td>
<td>8.3 ± 0.1</td>
</tr>
<tr>
<td>BPA (2,000) + NP (24)</td>
<td>5.9 ± 1.4</td>
<td>92.6 ± 2.8</td>
<td>8.5 ± 0.3</td>
</tr>
<tr>
<td>E2 (0.5)</td>
<td>10.1 ± 5.8</td>
<td>88.4 ± 5.2</td>
<td>8.9 ± 0.2</td>
</tr>
<tr>
<td>P value</td>
<td>0.815</td>
<td>0.710</td>
<td>0.083</td>
</tr>
</tbody>
</table>

Data expressed as mean ± standard error (n=3)
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 \( \mu g/L \) 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 \( \mu g/L \) BPA and 44.7 \( \mu g/L \) 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 results 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 \( \geq 355 \mu g/L \) BPA and \( \geq 23.5 \mu g/L \) 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 \( \mu g/L \) did not induce VTG synthesis, and 3,120 \( \mu g/L \) 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 \( \mu g/L \) BPA significantly increased plasma VTG in male zebrafish and juvenile rainbow trout, and both species exposed to 200 \( \mu g/L \) BPA concentration did not show induced VTG. The results (Kang et al., 2003) indicated that 24.8 \( \mu g/L \) NP concentration did not induce VTG synthesis but significantly increased in \( \geq 50.9 \mu g/L \) 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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